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DEVELOPMENT OF TWO
ALTERNATIVE MODELS OF
CAMPYLOBACTER JEJUNI INFECTION
THAT MORE CLOSELY MIMIC THE *IN VIVO*
ENVIRONMENT

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF
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Declaration

I declare that all the work and conclusions presented in this thesis are my own unless otherwise stated. All *ex vivo* organ culture (EVOC) experiments were performed at the University College London Institute of Child Health (ICH) in the laboratory of Dr Mona Bajaj-Elliott, initially assisted by Dr Lindsey Edwards. Analysis of the EVOC supernatants was carried out at ICH as well as at the London School of Hygiene and Tropical Medicine (LSHTM). All Vertical Diffusion Chamber (VDC) experiments were carried out at the LSHTM. Figure 3.11 contains ELISA data obtained by the ICH collaborators using supernatants from EVOC experiments performed by myself.

Abstract

Campylobacter jejuni is the leading cause of foodborne gastroenteritis worldwide, yet despite the organism's prevalence, relatively little is known about the mechanisms of pathogenesis. This is mainly due to the lack of a convenient small animal model of infection combined with certain inherent weaknesses with widely used *in vitro* models. The aim was to develop two improved models to study *C. jejuni* interactions with intestinal epithelial cells. The *Ex Vivo* Organ Culture (EVOC) model involves co-culturing *C. jejuni* with human intestinal biopsies. *C. jejuni* 11168H and 81-176 wild-type strains were demonstrated to induce the secretion of human beta-defensins 2 and 3 (hBD-2 and hBD-3). Furthermore, the supernatants of infected biopsies were demonstrated to contain significantly higher levels of IL-1 β , IL-6, IL-12 and IL-23 compared to uninfected controls. Experiments using 11168H *flaA* and *neuB1* mutants demonstrated that the induced defensin response was not due to host recognition of either flagellin or the terminal sialic acid residue of *C. jejuni* LOS. The Vertical Diffusion Chamber (VDC) model involves co-culturing *C. jejuni* with polarised human intestinal epithelial cells (IECs) with microaerobic conditions at the apical surface and aerobic conditions at the basolateral surface. Survival and integrity of the IECs under these conditions over 24 hours was demonstrated. Co-culture experiments under these conditions resulted in an increase in both *C. jejuni* interaction with and invasion of IECs. This was mirrored by an increased, polarised host innate immune response. Transcriptional analysis of aerobically and microaerobically co-cultured *C. jejuni* 11168H identified several genes that may play a role in these increased interactions. The levels of interaction and invasion of defined *C. jejuni* 11168H mutants with Caco-2 cells were analysed to identify bacterial factors that contribute to these increased interactions. Both 11168H *flaA* and *rpoN* mutants exhibited lower levels of interaction and invasion than the wild-type strain, suggesting the involvement of bacterial motility in the increased interactions under microaerobic conditions. The reduction in this increased interaction phenotype was more pronounced at shorter co-incubation times, suggesting that motility is particularly important during the early phases of interaction. The development of these two model systems should allow future

experiments to more accurately investigate host-pathogen interactions during *C. jejuni* infection of the human intestinal tract.

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Chapter 1

Introduction

1 Introduction

1.1 *Campylobacter jejuni*

1.1.1 Background

The earliest report of an organism that most likely belonged to the genus *Campylobacter* dates back to the early 20th century when John McFadyean and his associate Stewart Stockman were working on a government project investigating epizootic abortion in cattle and sheep (McFadyean, 1913, Skirrow, 2006). They reported “large numbers of peculiar organisms” that were “comma shaped” and classified them as *Vibrios* due to the morphology. In fact, they had most probably isolated *C. fetus*. In 1963, Sebald and Veron reclassified the organism into the new genus *Campylobacter* (campylo: Greek: crooked, bent). However, it took another 14 years until campylobacters were accepted as a causative agent of gastroenteritis in humans (Skirrow, 1977).

Despite the early discovery of campylobacters, the importance of the genus as a human intestinal pathogen remained unappreciated for a relatively long time. One reason for this may be that *C. fetus*, the species isolated by McFadyean and Stockman, is not a major human pathogen. Furthermore, isolation and *in vitro* culturing of *Campylobacter* is much more difficult than the culturing of other intestinal pathogens such as pathogenic *Escherichia coli* or *Salmonella* species. It was not until 1973, when techniques used to isolate *Campylobacter* species from ovine and bovine genital tracts were applied to a faecal sample from a young woman with acute diarrhoea, that the first successful isolation of the organism from a patient with gastroenteritis was reported (Butzler *et al.*, 1973). Further advances in culturing techniques (Bolton *et al.*, 1984, Karmali *et al.*, 1986) and rising awareness of the organism quickly lead to the finding that *Campylobacter* species were indeed important causative agents of gastroenteritis (Skirrow, 1977).

Since then, advances have been made in understanding the molecular basis of *Campylobacter* pathogenicity (Young *et al.*, 2007). However, the level of knowledge still lags behind that of other important enteric pathogens. This is

mainly due to the lack of suitable *in vitro* and *in vivo* models to study the organism. In light of the importance of the organism, developing a thorough, reproducible model of infection is essential to fully unravel the pathobiology of this important human pathogen.

1.1.2 Biology

Campylobacter jejuni is a member of the epsilon class of proteobacteria and belongs to the order of *Campylobacteriales*. This order also includes the genera *Wolinella* and *Helicobacter*. The genus *Campylobacter* contains several species, of which *C. jejuni* and *C. coli* are most commonly associated with human disease. In contrast, the first identified organisms from this genus, *Campylobacter fetus* spp. *fetus* and *Campylobacter fetus* spp. *venerealis* are implicated in disease in animals (Crushell *et al.*, 2004, Hoffer, 1981). *Campylobacters* are Gram-negative, flagellated bacteria and can be microscopically distinguished from other bacteria by their “spiral morphology and extremely rapid darting and spinning motions” (Butzler, 2004). The natural niche of *C. jejuni* is the intestinal tract of avian species, which can be colonised to exceptionally high numbers, with up to 10^{10} colony forming units (CFU) per gram of cecal contents having been found in chickens (Jacobs-Reitsma, 1995). The question of whether *C. jejuni* is an avian pathogen or a commensal has not been fully explored. There are several reports demonstrating an inflammatory response generated by cells of avian origin after *Campylobacter* challenge, both *in vivo* and *in vitro* (Borrmann *et al.*, 2007, Knudsen *et al.*, 2006, Smith *et al.*, 2008, Smith *et al.*, 2005, Byrne *et al.*, 2007). However, chickens colonised with *C. jejuni* have not been shown to develop symptoms analogous to those observed in humans.

1.1.3 Transmission

Several pathways of *C. jejuni* transmission have been identified to date. As the natural niche of the organism is the intestinal tract of birds and most commercial chicken flocks are colonised by *C. jejuni* (Blaser *et al.*, 1983b), handling and consumption of raw or undercooked (contaminated) chicken meat is thought to be the main source of *C. jejuni* infections in humans (Skirrow, 1991). Direct

transmission from animals has also been reported, with animal/carcass handlers such as farmers, butchers and veterinarians more prone to infection than people not in close contact with animals (Blaser *et al.*, 1983b). Other possible means of transmission such as consumption of unpasteurised milk (Blaser *et al.*, 1983b), contaminated river water (Schonberg-Norio *et al.*, 2004), contaminated drinking water (Vogt *et al.*, 1982) and even transmission by flies (Ekdahl *et al.*, 2005) have been proposed.

1.1.4 Epidemiology

Interestingly, the incidence of *Campylobacter*-related disease has increased during the last decade of the 20th century (Nachamkin, 2008). This is probably due in part to the implementation of better diagnostic methods and detection and may not directly reflect a real trend. However, the often self-limiting nature of the infection must also be considered, as this leads to many cases not being reported. Depending on the country, the true incidence is considered to be 8 to 30 times higher than the number of reported cases (Wheeler *et al.*, 1999, Samuel *et al.*, 2004). Intriguingly, *Campylobacter* epidemiology and incidence differ markedly between the developing and the developed world. In the developed world, reported numbers of *Campylobacter*-related disease range from zero to 396 cases per 100,000 people (Janssen *et al.*, 2008). People of all ages can be affected, although one peak occurs in young children and a second occurs in young adults (Nachamkin, 2008). Some data suggests that not only the incidence of *Campylobacter* infection, but also the prevalence of certain strains as causative agents, depend on age. It has been reported that older patients are generally infected with rarer serotypes than young patients (Miller *et al.*, 2005). This suggests that a basal level of immunity develops during life, but is not protective against all serotypes. In addition to an infection pattern changing with the age of the patient, *Campylobacter* infections in the developed world also show a seasonal pattern, with higher numbers appearing during the warmer months of the year (Altekruse *et al.*, 1999). This was originally attributed to different human behaviour in the summer months, such as having more barbeques and hence being subjected to a higher risk of cross-contamination (Kapperud *et al.*, 1992b). Recently, other factors such as the higher temperature

directly aiding the survival of the bacteria in the environment have also been implicated in causing the seasonal fluctuation (Louis *et al.*, 2005). In the developing world, *Campylobacter* related disease is mainly hyperendemic and usually associated with unhygienic conditions as well as close contact to animals (Rao *et al.*, 2001). Cases of enteritis peak in infants and young children and then decline rapidly. Case-control studies have suggested incidences of 40,000 to 60,000 cases per 100,000 children of less than 5 years of age. Additionally, asymptomatic carriage is more common in the developing world (Blaser *et al.*, 1985). This again suggests that infection leads to at least partial immune protection; if not against colonisation, then at least against disease. Whether this is due to a decreased interaction between the bacteria and the host cells or other factors is unknown. There is recent intriguing evidence from Vietnam suggesting that when living standards improve in a country, the presentation of *C. jejuni* related disease also changes (Dr Stephen Baker, Oxford University Clinical Research Unit, personal communication). This is in agreement with the differences observed between *C. jejuni* infections in the developing compared to the developed world.

1.1.5 Human disease

Human infection with *Campylobacter* occurs by the oral route. The first hurdle the bacteria have to overcome is the acidic environment of the stomach. Depending on the ingested dose and the buffering capacity of the food, most bacteria will be killed by the harsh acidic stomach environment. Survival of even a few organisms, however, has been shown to be sufficient to cause disease. The infectivity of *Campylobacter* is amongst the highest observed in intestinal pathogens, with infectious doses as low as 500 to 800 organisms sufficing to cause disease (Black *et al.*, 1988, Robinson, 1981). A probability for each individual CFU ingested to cause disease has been calculated at around 2% (Black *et al.*, 1988). Once the bacteria have passed through the stomach, the organisms establish themselves within the intestine resulting in infection, although asymptomatic carriage may also occur (Cawthraw *et al.*, 2000). In most immunocompetent people, infection is restricted to the intestinal tract, although systemic bacteraemia has been observed, ranging from 1.5 to 8 cases per 100,000

(Kapperud *et al.*, 1992a, Skirrow *et al.*, 1993). Intestinal disease commonly manifests itself as acute gastroenteritis after an incubation period of 1 to 7 days, which is longer than most other intestinal pathogens (Allos, 2001). The colonisation usually starts in the small bowel and subsequently moves to the colon and the rectum (Black *et al.*, 1988). The symptoms first encountered are mostly abdominal pain and cramping, often accompanied by fever, vomiting and headaches (Allos, 2001). This can make it difficult to distinguish early campylobacteriosis from influenza-like infections or even acute appendicitis (Blakelock *et al.*, 2003). Diarrhoea will start in parallel or slightly later than the above symptoms and can range from mild and watery to severe and bloody (Havelaar *et al.*, 2000, Blaser, 1997, Nachamkin, 2008). These differences in disease presentation have been attributed to both the infecting strain of *Campylobacter* as well as host factors (Black *et al.*, 1988). In an immunocompetent individual, the symptoms will usually subside within 3 to 7 days, although the bacteria may still be found in the faeces for several weeks (Kapperud *et al.*, 1992a). The disease is usually self-limiting, but can be treated with antimicrobials such as erythromycin or fluoroquinolones, if necessary (Anders *et al.*, 1982, Nachamkin, 2008).

1.1.6 Post-infectious sequelae

One important feature of *Campylobacter* infections is the possibility of developing post-infectious complications. These sequelae can be grouped into intestinal and non-intestinal manifestations.

1.1.6.1 Intestinal sequelae

Recent evidence suggests that *Campylobacter* infection may be linked to irritable bowel syndrome (IBS) (Smith *et al.*, 2007, Spiller *et al.*, 2000). IBS is characterised by abdominal pain associated with altered bowel habit after bacterial enteritis (Spiller, 2007). Inflammatory bowel diseases (IBDs) such as Crohn's disease have also been implicated to be linked with *C. jejuni* infection. (Berberian *et al.*, 1994, Weber *et al.*, 1992).

1.1.6.2 Extraintestinal sequelae

Although *C. jejuni* infection mainly leads to gastroenteric disease, the organism has also been linked to extra-intestinal sequelae. The most serious of these is Guillain-Barré syndrome (GBS), an autoimmune disease of the peripheral nervous system (Ang *et al.*, 2001, van Doorn *et al.*, 2008). Approximately 1 to 2 per 1000 people infected with *C. jejuni* go on to develop GBS (Mishu *et al.*, 1993). The disease is caused by molecular mimicry, as certain strains of *C. jejuni* possess lipo-oligosaccharide (LOS) structures that mimic glycans found on myelin sheaths in the peripheral nervous system (PNS). Infection with these strains leads to the development of auto-reactive antibodies, resulting in cross-reaction with and subsequent demyelisation of peripheral nerve cells (Yuki *et al.*, 2005). Mortality rates due to GBS in the developed world are around 2%, but most patients fully recover within 6-12 months (Nachamkin, 2008). Miller-Fisher syndrome, a sub-variant of GBS affecting mainly the nerves controlling eye movement, has also been implicated with *C. jejuni* infection (Yuki *et al.*, 1995). Reactive arthritis is another sequela associated with *C. jejuni* infection, although much less is known about this condition than about GBS. The incidence of reactive arthritis is believed to be around 4.3 per 100,000 individuals infected with *C. jejuni*. Small joints and knees are mostly affected, and a full recovery is usually made (Pope *et al.*, 2007). Even rarer, a few reports have implicated *C. jejuni* as a causative agent of haemolytic-uremic syndrome (Delans *et al.*, 1984), but this connection is still unclear and based only on a few studies. One common finding is that not all strains of *C. jejuni* cause the same symptoms and/or sequelae, and that even within the same strain there are differences, suggesting that host factors also play a part in determining the outcome of an infection.

1.2 Molecular basis of pathogenesis

The completion of the first genome sequence of *C. jejuni*, strain NCTC11168 in 2000 (Parkhill *et al.*, 2000), followed by that of other strains such as the highly virulent 81-176 (Hofreuter *et al.*, 2006) and RM1221 (Fouts *et al.*, 2005) greatly facilitated the search for molecular virulence determinants of *C. jejuni*. Availability of these genome sequences meant that genomic tools such as microarrays, transposon mutagenesis, targeted insertional mutagenesis,

proteomics and expression of recombinant proteins such as Green Fluorescent Protein (GFP) could now be utilised with this organism (Dorrell *et al.*, 2001, Hendrixson *et al.*, 2001, Karlyshev *et al.*, 2005b). It soon became clear, however, that the search would be hampered by several factors. Firstly, analysis of the sequences of *C. jejuni* genes revealed limited homology to those of other well studied enteric pathogens such as *Salmonella* species, pathogenic *E. coli* species or *Shigella* species, limiting the possibilities of homology-based studies. Secondly, even within the *C. jejuni* species, there is variation between the strains with regard to the presence of many genes (Dorrell *et al.*, 2001). Many of these differences are located within gene clusters that code for the biosynthesis and modification of surface structures such as the LOS, the capsular polysaccharide (CPS) or the flagellum, whereas genes involved in metabolism and regulation were more conserved between different strains. Interestingly, many genes displaying variability between strains are also those that have been shown to contain hypervariable sequences consisting of homopolymeric tracts (Parkhill *et al.*, 2000). This leads to differential expression of these genes as a result of phase variation, which in turn can lead to changes in phenotypic features such as ganglioside mimicry, motility or invasion (Linton *et al.*, 2000a, Gilbert *et al.*, 2002, Guerry *et al.*, 2006, Guerry *et al.*, 2002, Karlyshev *et al.*, 2002). Significant advances have been made in the last decade to solve the molecular and biochemical properties of *C. jejuni* pathogenesis; however the molecular basis for inducing diarrhoeal disease remains elusive. The following section will give an overview of the current knowledge of *C. jejuni* virulence factors.

1.2.1 Adhesion and invasion to host epithelial cells

It has long been acknowledged that entry into host cells is a way for pathogenic bacteria to evade the host's immune system and access a niche which the pathogen does not have to share with other organisms and that is potentially full of nutrients and other essential compounds. Colonic biopsies from patients with colitis and stool cultures positive for *Campylobacter jejuni* were shown to contain bacteria associated with the mucous layer on the surface of the epithelium as well as within the intestinal epithelial cells (IECs), suggesting that adherence and invasion of host IECs is one hallmark of *Campylobacter* infection

(van Spreeuwel *et al.*, 1985). These *in vivo* findings were followed by the discovery that *C. jejuni* isolates would adhere to and invade various epithelial cell lines *in vitro* (Konkel *et al.*, 1989, Szymanski *et al.*, 1995, Everest *et al.*, 1992). Multiple factors involved in bacterial adhesion and invasion have since been identified, both on the bacterial side as well as on the host side.

Several *C. jejuni* proteins have been termed adhesins, as their mutagenesis has led to a decrease in or loss of bacterial adhesion in *in vitro* tissue culture models. These proteins are usually surface associated and include the fibronectin binding protein CadF (Monteville *et al.*, 2003), Peb1a (Pei *et al.*, 1998), Peb3 (Rangarajan *et al.*, 2007), Peb4 (Asakura *et al.*, 2007) the surface lipoprotein JlpA (Jin *et al.*, 2001), the major outer membrane protein MomP (Moser *et al.*, 1997) and FlpA (Flanagan *et al.*, 2009). Apart from these adhesins, several other cellular structures and properties of *C. jejuni* have been implicated in adherence to and invasion of the epithelium such as surface charges and hydrophobicity (Walan *et al.*, 1988), the flagellum (see section 1.2.2), protein *N*-linked-glycosylation (see section 1.2.3.3), the secreted protein FlaC (Song *et al.*, 2004), the HtrA protease (Brondsted *et al.*, 2005), flagellin glycosylation (see section 1.2.3.4), the autotransporter CapA (Ashgar *et al.*, 2007), the CPS (see section 1.2.3.1), the global post-translational regulator CsrA (Fields *et al.*, 2008) and LOS (see section 1.2.3.2) However, the exact roles of most of these structures and proteins in bacterial adhesion and invasion remain poorly defined.

Most invasive bacteria manipulate the host cytoskeleton in one way or another during and after invasion, either by receptor ligand binding on the cell surface or by direct introduction of bacterial effector proteins into the host cytosol through specific secretion systems (for a review see (Pizarro-Cerda *et al.*, 2006). Some organisms such as *Shigella dysenteriae* will interfere with microtubule polymerisation/depolymerisation, while others such as *Salmonella* species will manipulate the actin filament assembly to gain entry into the host cells or localise themselves within a certain area of the host cell when internalised. In the case of *C. jejuni*, several studies have been conducted to analyse the pathway(s) used by the pathogen. An early study demonstrated that the chemical inhibition of actin filament formation by cytochalasin inhibited *C. jejuni* internalisation by IECs in a dose-dependent fashion (Konkel *et al.*, 1992). This suggested that actin filaments played an important role in the pathogen's internalisation into host

cells. The authors also commented that colchicine, an inhibitor of microtubule polymerisation, demonstrated dose-independent inhibitory effects on *C. jejuni* internalisation. An article published one year later, on the other hand, demonstrated that actin filament inhibitors showed only minimal effect on *C. jejuni* internalisation, whereas inhibitors of microtubule polymerisation had strong inhibitory effects on *C. jejuni* internalisation (Oelschlaeger *et al.*, 1993). Intriguingly, over the following years, two dogmas appear to have evolved, being carried by two of the authors of the initial reports. Konkel and colleagues pursued the actin filament path (Monteville *et al.*, 2003), while Kopecko and colleagues carried out further experiments to demonstrate the involvement of microtubules (Hu *et al.*, 1999, Kopecko *et al.*, 2001). Furthermore, another report suggested that inhibition of both actin filaments and microtubules led to a decrease of internalised *C. jejuni* (Biswas *et al.*, 2003, Biswas *et al.*, 2000). Whether these discrepancies between the experimental results reflect differences between the bacterial strains or the cell lines used or stem from different experimental procedures and beliefs is unclear. To date, no clear mechanism of *C. jejuni* cytoskeletal manipulation has been reported, and the means by which the bacteria trigger their uptake remain to be elucidated.

Once internalised, pathogenic bacteria have evolved numerous ways to avoid being degraded by the host defences. Some bacteria, such as *Shigella* species, remain inside a membrane-bound compartment but inhibit the host from degrading them. Others, such as *Listeria* species, escape from the compartment, replicate freely in the host cytoplasm, and from there invade neighbouring cells. In the case of *C. jejuni*, the intracellular fate of the bacteria is still relatively unclear. Recent evidence suggests that the organism survives within IECs inside *Campylobacter* containing vacuoles (CCV) and thereby avoids delivery to lysosomes (Watson *et al.*, 2008). These vacuoles have been postulated to localise in proximity to the Golgi apparatus. However, the exact role of the CCV in *Campylobacter* pathogenesis remains to be elucidated.

1.2.2 Flagellum

C. jejuni cells possess one or two polar, unsheathed flagella. The flagellum of *C. jejuni* has long been implicated in pathogenesis, as a mutant lacking the main

flagellar structural protein FlaA showed lower rates of invasion of IECs (Wassenaar *et al.*, 1991). This was initially believed to be the result of a lack of motility. Recent evidence, however, suggests that the flagella of *C. jejuni* do not exclusively have a role in bacterial motility, but also in the secretion of a set of proteins, termed the *Campylobacter* invasion antigens (Cia). These proteins were initially believed to be secreted by a type three secretion system (TTSS) due to amino acid sequence similarity with TTSS effector proteins (Konkel *et al.*, 1999a). However, the sequencing of the first *C. jejuni* strain NCTC11168 in 2000 revealed the absence of genes coding for a TTSS, suggesting that these proteins were secreted by a novel mechanism. As a result of this finding, the flagellum has been proposed as the system for secretion of the Cia proteins, as a functional flagellar apparatus is necessary for protein secretion to occur (Konkel *et al.*, 2004). Secretion of these proteins has been shown to be inducible *in vitro* by components of bile, most notably by the bile salt sodium deoxycholate (Malik-Kale *et al.*, 2008). Recently, a genetic screen revealed an *N*-terminal secretion sequence, which targets both flagellar and non-flagellar proteins to be secreted via the flagellum (Christensen *et al.*, 2009). The same screen revealed a novel protein of the Cia family termed CiaC which is involved in maximal invasion of host cells. However, the exact molecular mechanisms of action of these secreted virulence proteins remain to be elucidated. *C. jejuni* flagella have also been shown to be post-translationally modified by *O*-linked glycans (see section 1.2.3.4). Recent evidence using primary human intestinal tissue explants suggests that the flagella are involved in adhesion to the tissue epithelium as well as in bacteria-bacteria microcolony formation on the tissue (Haddock *et al.*, 2010). Once again, however, there is no consensus within the *Campylobacter* research community about the role of the flagellum and the Cia proteins during host-pathogen interactions. A recent article sheds doubt on the involvement of CiaB in host cell invasion by reporting the construction of a *ciaB* mutant in another *C. jejuni* strain (81-176) and demonstrating no difference in host cell invasion compared to the parental wild-type. (Novik *et al.*, 2010). The authors postulate that possible polar effects resulting in reduced motility led to the original findings and not a direct involvement of CiaB. Taken together, the exact role of *C. jejuni* flagella in secretion of effector proteins and host cell interactions remains to be fully elucidated.

1.2.3 Glycan structures

Sequencing of the first *C. jejuni* strain in 2000 revealed the presence of three major loci potentially involved in carbohydrate synthesis and transfer (Parkhill *et al.*, 2000). These include a CPS, a LOS moiety, as well as a system for post-translational modification of flagellar proteins. At about the same time, independent studies led to the identification of a fourth locus, demonstrated to be part of a protein *N*-glycosylation machinery (Szymanski *et al.*, 1999). Since then substantial analysis of all the four loci has been performed and all four have been confirmed functionally.

1.2.3.1 Capsular polysaccharide

CPSs are common in both Gram-positive and Gram-negative bacteria. CPS have been implicated in a variety of processes such as the prevention of desiccation, adherence of bacteria to each other and to other surfaces and resistance to complement (Roberts, 1996). As the CPS constitutes one of the outermost layers of the bacterial cell, this is also one of the first structures to come in contact with any host and has thus also been implicated in host recognition of pathogens. Until about 10 years ago, a high molecular weight polysaccharide detected on exopolysaccharide preparations of *C. jejuni* was believed to be the O-antigen repeat of a lipopolysaccharide (LPS). Analysis of the genome sequence, however, demonstrated the presence of two groups of genes containing *kps* orthologues flanking genes coding for proteins involved in sugar biosynthesis. The *kps* gene products are involved in CPS synthesis, transport and assembly and have been widely studied in *E. coli* (Silver *et al.*, 2001). Further functional studies provided evidence that these sugars were in fact a highly variable CPS (Karlyshev *et al.*, 2000). The sugar moieties were lost in bacteria with mutations in the *kpsM*, *kpsS* and *kpsC* genes; all three genes are orthologues of CPS export proteins in *E. coli*. Furthermore, the bacterial serodeterminant utilised in the standard serotyping procedure for *C. jejuni*, the Penner serotyping of heat stable antigens (Moran *et al.*, 1999), was shown to be the CPS (Karlyshev *et al.*, 2000). The presence of the CPS was also demonstrated by electron microscopy (Karlyshev *et al.*, 2001). Since its discovery, the *C. jejuni* CPS has been shown to be involved in virulence (Bacon *et al.*, 2001) and to be highly variable and

structurally diverse between serotypes (Karlyshev *et al.*, 2005a). Interestingly, one study suggests that the CPS is not involved in modulating an innate immune response nor in the resistance of *C. jejuni* against antimicrobial peptides (Zilbauer *et al.*, 2005). Another study demonstrated down-regulation of *C. jejuni* CPS in the presence of IECs (Corcionivoschi *et al.*, 2009). Recent work demonstrated the use of a CPS-conjugate vaccine as a protective compound against diarrhoeal disease in the new world monkey *Aotus nancymae* (Monteiro *et al.*, 2009).

1.2.3.2 Lipo-oligosaccharide

LPS or LOS decorate the outer membrane of many Gram-negative bacterial species. LPS consists of a lipid A moiety which anchors the LPS in the outer leaflet of the outer membrane, a core oligosaccharide usually containing one Kdo residue (3-Deoxy-D-manno-Octulosonic acid), and an O-antigen repeat consisting of polymerised oligosaccharides. The lipid A as well as the core-oligosaccharide are generally more conserved in structure and sugar content, while the O-antigen shows more variability between species and even strains. LOS differs from LPS in that it does not contain the O-antigen repeats and consists only of the Lipid A and the core oligosaccharide. LPS and LOS have been shown to be major antigenic and toxic compounds of Gram-negative bacteria, with the lipid A and the core oligosaccharide exhibiting the strongest immunogenic and toxic properties (Alexander *et al.*, 2001). Initially, the appearance of high molecular weight glycan structures in LPS/LOS preparations of *C. jejuni* was believed to indicate either that the organism possessed both LOS and LPS structures in the outer membrane (Blake *et al.*, 1993) or that the bacteria only possessed LOS structures that aggregated during the preparation steps (Mills *et al.*, 1985). The debate was ended by the discovery that the high molecular weight polysaccharide was in fact a CPS (see section 1.2.3.1). The LOS structures of several *C. jejuni* strains have since been determined, showing a strong degree of variability in the outer core region, whilst the inner core is more conserved amongst strains (Gilbert *et al.*, 2002). Additionally, the function of several genes involved in the synthesis and modification of the LOS glycan structures have been biochemically determined (Kanipes *et al.*, 2008). Consistent

with many other bacteria, the first sugar attached to the lipid A is Kdo residue. Two heptose sugars are then sequentially added by the action of the heptosyltransferases I and II, encoded by the *waaC* and *waaF* genes, respectively (Kanipes *et al.*, 2006, Oldfield *et al.*, 2002). The outer core is more variable between different strains of *C. jejuni*, and can even vary between different bacterial sub-populations of the same strain due to the phase variable expression of some of the glycosyltransferase genes. For example, phase variation of the β -1,3 galactosyltransferase WlaN in strain NCTC11168 can lead to the LOS either expressing a ganglioside GM₁ or a GM₂ mimicking structure (Linton *et al.*, 2000a). The outer core is also responsible for perhaps the most intriguing property of *C. jejuni* LOS, the structural mimicry of several human ganglioside glycans. This mimicry has been demonstrated to be the key cause of possible post-infectious GBS complications. The key glycan involved in this mimicry is a branched N-acetylneuraminic acid (NANA, sialic acid), a sugar rarely found in bacteria (Xiang *et al.*, 2006). Recent evidence suggests that LOS sialylation is an important determinant of IEC invasion *in vitro* (Louwen *et al.*, 2008) and recognition of the sialic acid residue by sialoadhesin, a lectin present on a subset of macrophages, has been demonstrated (Heikema *et al.*, 2010). Furthermore, stronger dendritic cell (DC) activation was detected after *in vitro* exposure to sialated purified LOS compared to unsialated LOS (Kuijf *et al.*, 2010). An association between sialated LOS and severe gastro-enteritis and reactive arthritis *in vivo* has also been demonstrated (Mortensen *et al.*, 2009). Taken together, *C. jejuni* LOS appears to play various roles during human infection.

1.2.3.3 N-linked glycosylation

N-linked glycosylation of proteins is a very common post-translational modification in eukaryotic cells, where the glycans play an important role in protein folding, oligomerization, quality control, sorting and transport (Helenius *et al.*, 2001). Until recently, it was believed that this process was restricted to eukaryotes and archaea. However, in 1999, Szymanski and co-workers discovered an operon in *C. jejuni* strain 81-176 that was similar to a LOS/LPS biosynthesis operon. When genes from the operon were mutated, however, no change in LOS electrophoretic mobility was detected. Instead, the

immunogenicity of several proteins was altered, indicating the presence of a protein modification system (Szymanski *et al.*, 1999). The locus was named *pgl* (protein glycosylation) and studied intensely in the following years. In an initial screen, 22 proteins were found to be modified with *N*-linked glycans and the structure of the glycan was determined (Young *et al.*, 2002). Subsequent functional transfer of the entire *C. jejuni* glycosylation locus into *E. coli* allowed for easier studying of the individual genes in the operon and opened up the novel possibility for generating recombinant glycoproteins (Wacker *et al.*, 2002). With regards to pathogenesis, the role of the *N*-linked protein glycosylation in *C. jejuni* has not been fully elucidated. It has been demonstrated that 81-176 *pglB* and *pglE* mutants, which lack the whole *N*-linked glycan due to either the absence of the oligosaccharyltransferase or the inability to synthesise the first end sugar, respectively, invade INT 407 cells at lower rates than the *C. jejuni* 81-176 wild-type strain and are impaired in the colonisation of the mouse gastrointestinal tract (Szymanski *et al.*, 2002). A 81116 *pglH* mutant, in which only a truncated oligosaccharide is synthesised and linked to the glycoprotein has also been shown to be less invasive of Caco-2 cells and impaired in chick colonisation (Karlyshev *et al.*, 2004). A screen of 22 *C. jejuni* 81-176 glycoprotein mutants revealed one gene that led to a loss of invasiveness of INT 407 cells if mutated (*Cjl496*) (Kakuda *et al.*, 2006). However, the role of the glycan in this phenotype is still unclear, as complementation with an unglycosylatable variant of the protein restored the phenotype. The same is true for the glycoprotein encoded by *znuA*, a component of a putative zinc ABC transport system (Davis *et al.*, 2009). Despite detailed knowledge of the *N*-glycosylation machinery, the role of glycoproteins in the biology of *C. jejuni* still remains to be fully elucidated.

1.2.3.4 *O*-linked glycosylation

As opposed to *N*-linked glycosylation, *O*-linked glycosylation of proteins in *C. jejuni* is more diverse and heterogenous, both genetically and phenotypically. Logan and co-workers first discovered the presence of post-translationally modified serine residues on the flagellin protein of *C. coli* (Logan *et al.*, 1989). The glycan most frequently attached to the flagellin proteins was determined to be pseudaminic acid, with added heterogeneity generated through substitution of

certain groups (Thibault *et al.*, 2001). The biosynthesis of the flagellar glycans and their transfer to flagellin have since been analysed in some depth by investigation of strains mutated in genes coding for sugar modifying and transferring enzymes as well as by targeted metabolomics studies (McNally *et al.*, 2006, Goon *et al.*, 2003, Vijayakumar *et al.*, 2006). Through a functional metabolomics study, a novel glycan moiety attached to the flagellin of *C. coli*, legionaminic acid, was discovered (McNally *et al.*, 2007). Regarding the biological function of these modifications, it has been proposed that the *O*-linked glycosylation is required for efficient assembly and function of the flagella, as mutants unable to glycosylate their flagella either through site-directed mutagenesis of acceptor serine residues or through mutations of the glycan synthesis/transfer enzymes have been demonstrated to be non-motile (Ewing *et al.*, 2009, Guerry *et al.*, 2006). Flagellin glycosylation has also been shown to be involved in the colonisation of chickens (Howard *et al.*, 2009).

1.2.4 Cytolethal distending toxin (CDT)

Bacterial CDTs belong to the AB₂-type toxins and generally consist of three subunits, CdtA, CdtB and CdtC (Ge *et al.*, 2008). CdtA and CdtC are binding proteins that act as carriers to deliver the catalytic subunit, CdtB, into host cells (Smith *et al.*, 2006). Once inside the cells, CdtB exhibits DNase I-like activity and induces limited DNA damage such as double strand damage, leading to the activation of DNA repair responses and ultimately an arrest of the cell cycle at the G₂/M phase (Lara-Tejero *et al.*, 2001, Whitehouse *et al.*, 1998). The presence of a CDT in *C. jejuni* was determined relatively early on in *C. jejuni* research (Johnson *et al.*, 1988) and the *Campylobacter* CDT has in fact been useful in studying the function of this class of toxin (Lara-Tejero *et al.*, 2000, Lara-Tejero *et al.*, 2001). With regards to pathogenesis, *C. jejuni* CDT has been shown to be involved in mediating the release of interleukin 8 (IL-8) from IECs (Hickey *et al.*, 2000, Zheng *et al.*, 2008), to promote DNA repair responses in human cells (Hassane *et al.*, 2003), to be involved in gastroenteritis in NFκB-deficient mice (Fox *et al.*, 2004), and to induce cell death in human monocytic cells (Hickey *et al.*, 2005). Additionally, the *cdtABC* genes have been used as targets for multiplex PCR reactions aimed at detecting and identifying *C. jejuni*,

C. coli and *C. fetus* (Asakura *et al.*, 2008, Fernandes *et al.*, 2009). Recently, the presence of CDT proteins in *C. jejuni* outer membrane vesicles (OMVs) has been demonstrated, suggesting that these vesicles may be one pathway utilised by *C. jejuni* to deliver the toxin to the host (Lindmark *et al.*, 2009).

1.3 Host response to *C. jejuni* infection

1.3.1 Innate Immunity

The epithelial lining of the intestine acts as the first physical barrier between any intestinal pathogen and the host. However, the cells of the epithelium have a further role in defence against pathogens; they are capable of sensing the presence of a pathogenic microorganism and transmitting this to other cells of the innate immune system. Epithelial cells possess mechanisms that can detect both extracellular pathogens as well as organisms that have gained entry into the cells through different sets of pathogen recognition receptors (PRRs) (Sanderson *et al.*, 2007). Of the extracellular receptors, the Toll-like receptor (TLR) family is amongst the best described (Kumar *et al.*, 2009). TLRs are grouped into families depending on the recognised microbe-associated molecular pattern (MAMP) and are at the beginning of signalling cascades, involving nuclear targeting of the transcription factor NF- κ B. (Bryant *et al.*, 2009) TLR4 for example recognises bacterial LPS or LOS while TLR5 detects the flagellar subunit protein flagellin (Vijay-Kumar *et al.*, 2009). In the case of *C. jejuni*, it has been demonstrated that flagellin is a poor activator of TLR5 signalling (Watson *et al.*, 2005, Johanesen *et al.*, 2006). The site recognised by TLR5 has recently been identified and found to be absent from *C. jejuni* flagellin, thus leading to the lack of recognition (Andersen-Nissen *et al.*, 2005). With regards to TLR4, recent work demonstrated that bone-marrow derived dendritic cells (BM DCs) from mice from a TLR4-deficient background were less responsive to *C. jejuni* challenge than wild type BM DCs, suggesting a role for this receptor in the recognition of the pathogen (Rathinam *et al.*, 2009). The same study also identified the involvement of TLR2 which recognises bacterial lipoproteins in the BM DC response to *C. jejuni*.

Furthermore, the myeloid differentiation factor 88 (Myd88) protein, which is involved in most TLR-dependent signalling, has been implicated in innate

immune response to *C. jejuni* in mouse BM DCs and in a Myd88 knockout mouse colonisation study (Rathinam *et al.*, 2009, Watson *et al.*, 2007). However, human IECs have been shown to respond to *C. jejuni* challenge in a Myd88-independent way (Friis *et al.*, 2009), suggesting that several pathways are utilised to trigger a response.

Downstream of the PRRs, a variety of signalling cascades transmit and amplify the signal. These cascades are made up of a plethora of kinase proteins, and several of these have been implicated in signalling in response to *C. jejuni* challenge. As early as 1996, a role for the phosphatidylinositol-3 kinase (PI-3 kinase) in IEC entry and signalling of *C. jejuni* was demonstrated (Wooldridge *et al.*, 1996). The surface lipoprotein JlpA has been demonstrated to interact with the surface exposed heat shock protein 90alpha and lead to activation of the p38 MAP kinase in IECs (Jin *et al.*, 2001). A further study demonstrated that the ERK protein kinase was activated alongside the p38 MAP kinase in response to *C. jejuni* challenge (Watson *et al.*, 2005). Additionally, the Jun N-Terminal kinase (JNK) was shown by two studies to be activated in response to *C. jejuni* challenge (MacCallum *et al.*, 2005a, Chen *et al.*, 2006).

Receptors of the nucleotide oligomerization domain (NOD) family are specialised intracellular PRRs involved in regulation of an innate immune response (Franchi *et al.*, 2009). Similar to the TLR receptors, they stand at the head of a signalling cascade that involves NF- κ B signalling. Recently, NOD1, but not NOD2, was demonstrated to play an important role in the host recognition of *C. jejuni* in IECs (Zilbauer *et al.*, 2007). When NOD1 was targeted with small interfering RNA (siRNA), an increase in the number of intracellular *C. jejuni* was detected, highlighting a role for NOD1 in the defence against the pathogen.

Once a pathogen is detected by a PRR, in most cases, downstream signalling involves activation of the transcription factor NF- κ B. This in turn leads to expression of several innate immunity genes. NF- κ B activation in IECs has been shown to occur in response to *C. jejuni* challenge by several studies (Zheng *et al.*, 2008, Chen *et al.*, 2006, Mellits *et al.*, 2002, Jin *et al.*, 2001, Johanesen *et al.*, 2006). Furthermore, an NF- κ B deficient mouse has been demonstrated to exhibit gastroenteritis when infected with *C. jejuni* (Fox *et al.*, 2004), and NF- κ B

activation has been demonstrated in *C. jejuni* infected gnotobiotic and IL-10 deficient mice exhibiting gastroenteritis by promoter fusion (Lippert *et al.*, 2009).

Once the pathogen has been detected by the epithelium, the information must be transmitted to other parts of the innate immune system. Small signalling peptides are the carriers of such signals; the chemokine Interleukin 8 (IL-8) for instance is a chemoattractant, recruiting immune cells such as neutrophils to a site of infection. IL-8 signalling has been clearly established as one of the responses of intestinal epithelia to *C. jejuni* infection (Hickey *et al.*, 1999). The secretion of IL-8 has been shown to be dependent on the presence of CDT (Hickey *et al.*, 2000, Zheng *et al.*, 2008) and requires contact between the bacteria and the IECs (Watson *et al.*, 2005). It has also been demonstrated that the secretion of IL-8 by a polarised monolayer of IECs is directional, with most of the IL-8 found on the basolateral side of the monolayer (Chen *et al.*, 2006). Furthermore, not all IEC lines respond alike to *C. jejuni* challenge, with some lines secreting high quantities of IL-8, whilst others only secrete low levels (MacCallum *et al.*, 2006). Recently, IECs have been demonstrated to secrete Interleukin-6 (IL-6) (Friis *et al.*, 2009) and tumour necrosis factor alpha (TNF- α) (Zheng *et al.*, 2008) in response to *C. jejuni* challenge. Additionally, the intestinal epithelium has been shown to not only sense the presence of *C. jejuni* and relay the signal to other immune cells; evidence demonstrated that IECs produced antimicrobial peptides of the defensin family in response to *C. jejuni* challenge (Zilbauer *et al.*, 2005). Transcription as well as secretion of both hBD-2 and hBD-3 was demonstrated in IECs infected *in vitro* with *C. jejuni*, and both antimicrobial peptides exhibited potent bactericidal activity against *C. jejuni*.

Once *C. jejuni* has crossed the epithelial barrier, the bacteria come into contact with other cells of the innate immune system such as dendritic cells (DCs), macrophages and neutrophils. Human DCs have been shown to undergo maturation and produce cytokines in response to *C. jejuni* challenge (Hu *et al.*, 2006). Furthermore, DCs in rectal mucosal biopsies of patients with acute *Campylobacter* colitis showed higher prevalence of ultrastructural features of activation than control samples (Pulimood *et al.*, 2008). Mouse DCs have also been shown to be activated by *C. jejuni* and to signal via the pathways mentioned above (Rathinam *et al.*, 2008, Rathinam *et al.*, 2009). Recently, the human

Macrophage Galactose-type lectin (MGL), which is mainly expressed on immature DCs and a specific subset of macrophages has been shown to bind to the *C. jejuni* N-glycan and has been implicated in possible immunosuppression (van Sorge *et al.*, 2009).

Neutrophil infiltrates have been detected in biopsies of ferrets infected with *C. jejuni* (Fox *et al.*, 1987), in the cecum and colon of infected gnotobiotic pigs (Boosinger *et al.*, 1988), and in intestinal biopsies of infected *Macacca nemestrina* monkeys (Russell *et al.*, 1989). However, the exact role of neutrophils in *C. jejuni* infection remains to be elucidated and no *in vitro* data of neutrophil interactions with *C. jejuni* has been obtained to date.

Unlike neutrophil-*C. jejuni* interactions, interactions between macrophages and *C. jejuni* have been studied in more depth. Macrophages from different organisms such as humans, mice, chickens and guinea pigs, as well as primary and “immortalised” macrophages have been used. Intriguingly, however, the reported results are not always consistent. The first report of an *in vitro* co-culture of *C. jejuni* with a murine macrophage line, primary murine peritoneal macrophages as well as human peripheral blood monocytes demonstrated that *C. jejuni* was readily phagocytosed by all macrophages (Kiehlbauch *et al.*, 1985), with human macrophages ingesting “more rapidly and vigorously” than murine macrophages. The authors also demonstrated survival of the bacteria within the macrophages for up to 7 days. Co-culturing of chicken peritoneal macrophages and *C. jejuni* revealed that these cells were also able to phagocytose the bacteria and that phagocytosis was increased by pre-incubating the bacteria with an antiserum (Myszewski *et al.*, 1991). The authors reported that the chicken-derived macrophages managed to kill all bacteria within the experimental period of maximum 6 h. However, another study using human peritoneal monocytes/macrophages implicated host factors in the killing of *C. jejuni* by the macrophages, as cells from 10% of the donors were able to phagocytose, but not kill the bacteria (Wassenaar *et al.*, 1997). A further study using the human monocytic cell line THP-1 demonstrated the occurrence of caspase-1 independent apoptosis and induction of Interleukin 1 beta (IL-1 β) (Siegesmund *et al.*, 2004). The most recent study using bone marrow-derived (BMDM) murine macrophages demonstrated complete killing of *C. jejuni* by the macrophages after 24 h (Watson *et al.*, 2008).

1.3.2 Adaptive immune response

C. jejuni infection has been demonstrated to lead to an increase of serum titres of anti-*Campylobacter* IgG, IgM and IgA antibodies (Cawthraw *et al.*, 2000, Strid *et al.*, 2001). However, the kinetics of the three antibody classes differ. While IgM and IgA were detected during the acute phase of the infection (up to two months after infection) and then declined, IgG antibodies can persist for months or even years after an infection. Re-infection of previously infected adults has shown that the previous infection was able to protect from disease, but not from colonisation, suggesting a role for humoral immunity in determining the outcome of the disease (Black *et al.*, 1988). A further study demonstrated an age-dependent rise in serum-specific antibodies in developing countries, where *C. jejuni* infections are common from an early age. This was shown to lead to successively milder symptoms with each infection (Taylor *et al.*, 1993).

1.4 Models used to study *C. jejuni* pathogenesis

In order to understand how a pathogenic organism causes disease in humans, the availability of a good model of the disease is the most essential factor. Over the past decades, multiple pathogenic organisms have been studied using a variety of *in vivo* animal models such as rodents, combined with *in vitro* culturing models. In the case of *C. jejuni*, the search for convenient models of the disease has proven to be more difficult than for other organisms, which has in turn led to a lag in the knowledge regarding *C. jejuni* virulence factors and key host responses to infection compared to other enteric pathogens.

There is a clear need for the development of new models to study *C. jejuni in vivo* as well as *in vitro*, to further understand the molecular pathogenesis of this important microorganism.

1.4.1 *In vivo* models used to study *C. jejuni*

When developing an animal model to study a human pathogenic microorganism, a set of key points need to be met to determine whether the model will be suitable for the planned studies. These points are:

- Reproduction of the disease found in humans
- Commercial availability
- Ease of laboratory handling
- Good lifespan/generation time
- Availability of genetic tools/mutants
- Availability of other tools such as sera
- Ethical agreement

1.4.1.1 Humans

The best model of infection for a human pathogen would always be humans themselves. A few human experimental studies have been performed, demonstrating that humans can indeed be experimentally infected with *C. jejuni* (Black *et al.*, 1988, Prendergast *et al.*, 2004). However, the main concern regarding the use of a human challenge model is the possibility of GBS. The possibility that some of the volunteers may develop this serious post-infectious sequela renders the model ethically questionable. Recently, a set of *C. jejuni* strains that were found to lack the genes required to synthesise the LOS structures involved in inducing GBS were assessed as potential vaccination candidates (Poly *et al.*, 2008). The strains were shown to lack the ganglioside mimicking glycans on their surface and one strain was evaluated in a human challenge study (Tribble *et al.*, 2009). These strains may provide a novel, safer tool to study the complex interaction of the bacterium with the human host. However, whilst lowering the ethical constraints due to the inability to cause GBS, the strain still does not address the problems of generation time, “handling” and availability of genetic tools for the human model of *C. jejuni* infection.

1.4.1.2 Non-human primates

Non-human primates are considered good models for infection studies with human pathogens, as they are more closely related to humans than rodents or other mammals. A couple of studies have been performed using different species of rhesus monkey. A first study using young *Macaca mulatta* demonstrated a mild disease outcome, with short lasting diarrhoea, prolonged excretion of bacteria, and bacteraemia for the first 2 to 3 days after infection (Fitzgeorge *et al.*, 1981). Another study with young *Macaca nemestrina* monkeys demonstrated a more severe outcome of the disease, including acute diarrhoeal illness, with fluid diarrhoea, bloody stools and faecal leukocytes (Russell *et al.*, 1989). Re-infection of the animals led to only mild diarrhoea, strengthening the hypothesis of a role for the adaptive immune system in protection towards disease caused by

C. jejuni. However, despite the promising preliminary results, the rhesus monkey remains a suboptimal ideal model, mainly due to the fairly complicated handling and housing requirements and the absence of genetic tools.

1.4.1.3 Dogs

Two reports discuss dogs as a potential model to study *C. jejuni* mediated disease. In the first, gnotobiotic Beagle puppies were orally inoculated with *C. jejuni* from human and canine origin and displayed transient listlessness, lack of appetite and mild diarrhoea (Prescott *et al.*, 1981). In the second, puppies were orally inoculated and displayed mild disease; however no penetration of IECs was detected (Macartney *et al.*, 1988). However, as dogs have been reported to be natural carriers of *C. jejuni*, they are not an ideal model, and precautions such as keeping the animals germ-free are necessary (Moreno *et al.*, 1993).

1.4.1.4 Pigs

As the digestive tracts of pigs and humans are not that different, pigs have been considered to be a potentially attractive model to study *C. jejuni*. It has been demonstrated that gnotobiotic piglets can be readily infected orally with *C. jejuni* (Boosinger *et al.*, 1988, Vitovec *et al.*, 1989). The cecum and colon of infected pigs showed oedema, neutrophil infiltration and sloughing of IECs from the mucosa. Furthermore, colostrum-deprived newborn piglets have also been successfully experimentally infected with *C. jejuni*. The animals developed clinical symptoms and histopathological lesions similar to humans infected with *C. jejuni* (Babakhani *et al.*, 1993a). Pigs seem to constitute a reasonably good, reproducible model system to study *C. jejuni*. However, to date only a few studies have used pigs, most likely due to the complex housing and handling requirements.

1.4.1.5 Ferrets

Ferrets have been used by several groups as a potential model for *C. jejuni*. Weanling ferrets inoculated orally with *C. jejuni* became infected after 3 days

and had bile-tinged, liquid faeces with excessive mucous and blood (Fox *et al.*, 1987). Another study using juvenile and adult animals demonstrated a disease spectrum similar to humans, with diarrhoea ranging from mild to moderate which was spontaneously resolved in a few days (Bell *et al.*, 1990). A recent study explored the pathology and immune response of *C. jejuni* infected ferrets in more detail, highlighting vigorous systemic and mucosal immune responses to *C. jejuni* challenge (Nemelka *et al.*, 2009). Taken together, these data suggest that ferrets are a plausible model for studying *C. jejuni*. However, similar to the pig model, only relatively few studies have been conducted using ferrets, most likely due to the laborious handling and lack of genetically defined mutants and reagents.

1.4.1.6 Chickens

Chickens as one of the natural hosts of *C. jejuni*, have been assessed for their potential as a model. There remains an ongoing discussion whether *C. jejuni* is a chicken commensal or a pathogen. Most studies conducted with chickens have been aimed to decipher factors relevant for colonisation rather than pathogenesis, potentially with the aim of lowering the burden of infected chickens on farms. A *C. jejuni flaA* and a *C. coli sodB* mutant have been demonstrated to exhibit defects in chicken colonisation, suggesting an involvement of the major flagellar protein FlaA and the superoxide dismutase SodB in the colonisation of chickens (Nachamkin *et al.*, 1993, Purdy *et al.*, 1999). A large signature tagged mutagenesis project led to the discovery of 22 genes involved in commensal colonisation of the chicken gastrointestinal tract (Hendrixson *et al.*, 2004). Recent evidence suggests that there may be a controlled inflammatory process occurring during colonisation of the chicken intestine (Smith *et al.*, 2008), and that *C. jejuni* differentially activates TLRs in chickens compared to humans (de Zoete *et al.*, 2009). However, as the chickens colonised with *C. jejuni* do not suffer from any symptoms similar to those encountered in humans, the feasibility of a chicken model remains questionable.

1.4.1.7 Mice

Small rodents, particularly mice, are amongst the most widely used models of infection to study human pathogens. Successful mouse models have been developed for many bacteria, including *Salmonella* spp. (Hapfelmeier *et al.*, 2005), enteropathogenic *E. coli* using the related mouse pathogen *Citrobacter rodentium* (Mundy *et al.*, 2005), *Vibrio cholera* (Klose, 2000) and *Yersinia enterocolitica* (Oellerich *et al.*, 2007). The mouse has several major advantages over all of the other animal models described above. The generation time is low, handling is relatively simple, there are many knockout strains available, as well as a whole range of sera and other tools to study the host response to a pathogen. In 1983 a first mouse infection study with *C. jejuni* reported that the bacteria readily colonised mice, leading to a brief spike of bacteraemia 10 minutes after oral infection, but did not lead to any signs of illness, even though the challenged mice developed into chronic excretors (Blaser *et al.*, 1983a). This finding was confirmed by several further studies, suggesting that *C. jejuni* rapidly colonised mice, but did not appear to lead to the development of disease (Lee *et al.*, 1986). Another study further demonstrates the difficulties encountered when using mice as a model for *C. jejuni*. Although the authors succeeded in generating symptoms of disease in mice using *C. jejuni*, a very high infection dose of 10^8 to 10^9 CFUs injected intraperitoneally into mice pre-treated by injection with iron dextran was necessary for the development of disease (Stanfield *et al.*, 1987). Mice that had not been pre-treated with iron dextran did not develop the disease. Furthermore, the levels of colonisation varied substantially between different replicate mice. More recently, several knockout mutant mouse strains have been tested for their suitability as a model for *C. jejuni* pathogenesis. Mice deficient in NF κ B have been reported to develop moderately severe gastritis and duodenitis when challenged with *C. jejuni*, compared to mild gastritis in the wild type parental mice (Fox *et al.*, 2004). Furthermore, the authors highlighted a role for the CDT as an important virulence factor in these mice. Another group reported the use of congenic interleukin-10 (IL-10) deficient mice and demonstrated the development of severe typhocolitis in the mutant mice compared to the wild type

parent strain. However, as the wild type mice showed similar rates of colonisation but no symptoms, the authors hypothesised that colonisation of the gastrointestinal tract is necessary but not sufficient for the development of enteritis (Mansfield *et al.*, 2007). The same year, Watson and co-workers reported the use of a MyD88 deficient mouse model (Watson *et al.*, 2007). They demonstrated that the mutant mouse was readily colonised by *C. jejuni*, but not by bacterial strains deficient in known virulence factors. Furthermore, they hypothesised that Natural resistance-associated macrophage protein one (Nramp1)-deficiency leads to an increased mouse susceptibility to systemic infection, as Nramp1-deficient mice were less able to clear *C. jejuni* after intraperitoneal inoculation. This finding was confirmed by another study, further strengthening the hypothesis that Nramp1 is critical in host resistance to *C. jejuni* (Champion *et al.*, 2008). The most recent finding using gnotobiotic IL-10 deficient mice demonstrated development of severe ulcerating colonic inflammation and bloody diarrhoea in response to *C. jejuni* infection (Lippert *et al.*, 2009). Three other reports demonstrate disease and systemic spread of *C. jejuni* after intranasal challenge, although it is debatable whether a lung model is suited for an enteropathogen (Baqar *et al.*, 1996, Al-Banna *et al.*, 2008b, Al-Banna *et al.*, 2008a). Taken together, it can be stated that to date, even though several mouse models have been evaluated, there still remains the need for further development, as none of the models has led to any significant advances in our understanding of the molecular basis of pathogenicity of *C. jejuni*. Nevertheless, the mouse model remains the most promising *in vivo* model.

1.4.2 *In vitro* models used to study *C. jejuni*

An alternative to using *in vivo* models to understand how a pathogenic organism causes disease is the use of *in vitro* systems. As the intestinal epithelium is the part of the host with which the bacteria mainly interact whilst in the gut, there is a strong rationale to use a co-culture method of *C. jejuni* with IECs. These can either be primary IECs harvested from intestinal tissue sections or “immortalised” cell lines that have usually been derived from carcinomas.

1.4.2.1 Primary cells

The advantage of primary cells is that they are freshly isolated from the host and are not cultivated and lab-adapted, thus representing the *in vivo* state much more closely than cell lines. On the other hand, isolation of primary cells can be difficult and it is not always easy to culture them long enough to perform experiments. Primary cells from different organisms have been assessed in *in vitro* co-culturing experiments. Babakhani and co-workers utilised primary swine IECs from 6 hour old piglets and demonstrated invasion of the IECs by *C. jejuni* (Babakhani *et al.*, 1993b). Primary cells of chickens from different organs of the bird have been used in order to try to understand the differences between humans and chickens infected with *C. jejuni*. Using avian primary chick kidney cells, Smith and co-workers demonstrated expression of pro-inflammatory cytokines and chemokines in response to *C. jejuni* (Smith *et al.*, 2005). Another study supported this finding by demonstrating the induction of IL-8 in primary intestinal chick cells (Borrmann *et al.*, 2007). Two other studies showed that *C. jejuni* is capable of invading primary chick embryo IECs and primary adult chicken IECs (Li *et al.*, 2008, Byrne *et al.*, 2007). Intriguingly, these studies suggest that *C. jejuni* interacts with avian cells in a very similar way to human cells and that the cells respond in turn. It remains to be fully elucidated why the outcome of colonisation/infection is so different in chickens and humans. Primary cells of human origin have been used in very few studies, most likely because they are more difficult to obtain than animal cells. Four studies used an *ex vivo* organ culturing (EVOC) model to co-culture *C. jejuni* with human intestinal biopsies. Using this model, it was demonstrated that *C. jejuni* was able to adhere to the biopsies *in vitro* (Grant *et al.*, 2006) and IL-8 secretion as well as mitogen-activated kinase activation were reported in response to infection in human biopsies (MacCallum *et al.*, 2006, MacCallum *et al.*, 2005a). Recent data from the same group demonstrated the importance of flagella in the interaction of *C. jejuni* with human primary intestinal tissue and showed that the bacteria formed distinct microcolonies on the ileal tissue before spreading out and forming a biofilm (Haddock *et al.*, 2010).

1.4.2.2 Immortalised epithelial cell line models

The most common type of *in vitro* model used to study host-pathogen interactions is the epithelial cell line model. These cell lines are usually derived from a carcinoma and are available from a wide range of organisms and a wide range of tissues. The American Type Culture Collection (ATCC) for example offers approximately 1100 tumour cell lines from a variety of species (www.atcc.org/Portals/1/TumorLines.pdf). These cell lines have the advantage that they are readily available and often relatively easy to cultivate. The most common type of cells used for studying *C. jejuni* host-pathogen interactions are human cell lines stemming from the intestinal epithelium, although cells from other organs have also been used (Table 1.1).

Table 1.1: Cell lines used to study *C. jejuni* *in vitro*.

Cell line	Organism	Organ	Forms polarised monolayer
Caco-2	Human	Colon	Yes
HeLa	Human	Cervix	No
HEp-2	Human	Cervix	No
Int 407	Human	Intestine	No
HT29	Human	Colon	Yes
T84	Human	Colon	Yes
MDCK	Dog	Kidney	Yes

Adapted from Friis *et al.* 2005

The first evidence that *C. jejuni* invaded epithelial cells of intestinal and non-intestinal origin *in vitro* was obtained around 1990 (Everest *et al.*, 1992, De Melo *et al.*, 1989), followed by demonstrations that the IECs responded to *C. jejuni* challenge (Hickey *et al.*, 1999). Most of the findings summarised in section 1.2 were elucidated using this type of model. However, the fact that several different cell lines have been used combined with contradictory results obtained by different research groups, suggests that the 2D monolayer model is not totally satisfactory. The cell line used represents a particular research group’s preference rather than an established rationale for using that line. Furthermore, the rate of

interaction of the bacteria with the IECs is substantially lower than that of other enteropathogens such as *Salmonella* spp. In 2005, Friis and co-workers reviewed the use of “*in vitro* cell culture models for investigating *Campylobacter* invasion mechanisms” (Friis *et al.*, 2005). The authors highlighted several difficulties and variables encountered when using this type of model such as the multiplicity of infection (MOI) or the bacterial strain used. A number of suggestions were proposed to tackle these variables such as establishing the effects of different MOIs for each cell line and mathematically evaluating these effects. Following these suggestions might lead to an optimisation of the classical 2D monolayer model of infection, increasing reproducibility and strengthening the model. However, as things stand to date, there is no generally accepted protocol for *in vitro* co-culturing of *C. jejuni* with epithelial cells.

There is an additional fact that must be considered when using this model to study *C. jejuni* interactions with epithelial cells. In order to assure survival of the IECs, the co-culturing must be performed under aerobic conditions. This does not affect bacteria such as *Salmonella* or *E. coli*, as these organisms are facultative anaerobes that thrive under atmospheric oxygen conditions. On the other hand, *C. jejuni* is a microaerophilic organism. Although *C. jejuni* has been shown to possess mechanisms to survive oxidative stress such as the superoxide dismutase (Pesci *et al.*, 1994, Purdy *et al.*, 1994), catalase (Day *et al.*, 2000), polyphosphate kinase 1 (Candon *et al.*, 2007) and other stress response proteins, it is probable that the bacteria will behave differently under atmospheric oxygen conditions than in a microaerobic or even anaerobic environment. Furthermore, the environment in the human intestine is probably almost anaerobic. There is therefore a strong possibility that the aerobic nature of the classical monolayer IEC culture model is not ideal to study the pathogenesis of the microaerophilic *C. jejuni* and may have effects on the pathogen’s behaviour leading to contradictory observations.

The interaction of *Helicobacter pylori*, another microaerophilic bacterial species related to *C. jejuni*, was studied under microaerobic and aerobic conditions (Cottet *et al.*, 2002). This study was performed by using a Vertical Diffusion

Chamber (VDC) or Ussing Chamber (UC), which was originally developed by Grass and Sweetana to study the transport of substances across membranes (Grass *et al.*, 1988). The design of this system was adapted such that when IECs grown on permeable plastic supports (Snapwells™) are inserted into the chamber, the cells act as a barrier, creating an apical and a basolateral compartment. The conditions in the two compartments can then be individually adjusted; both the content of the compartment (medium, bacteria and other substances) and the gas composition can be individually manipulated. *H. pylori* was demonstrated to adhere to the IECs in a greater number under microaerobic conditions and the innate immune response of the IECs was increased due to this greater interaction. Furthermore, an increased expression of the *H. pylori* urease was detected under microaerobic conditions. As *H. pylori* and *C. jejuni* are both microaerophilic, we hypothesised that applying the VDC model to *C. jejuni* will analogously increase the interactions of the bacteria with the IECs.

A further limitation of the classic IEC-bacteria co-culturing model is the presence of only one type of cells, the IECs. A polarised monolayer of IECs may represent a major part of the human intestine an invading pathogen encounters; however, the morphology of the gut wall as well as the cellular content is much more complex than a monolayer. Areas termed Peyer's patches, aggregation of lymphoid tissue containing a type of cell called M cells, are present in the lowest portion of the small intestine, the terminal ileum. Moreover, it has been shown that another type of immunomodulatory cell belonging to the DC class penetrates the epithelium and samples bacteria (Rescigno *et al.*, 2001). As these types of cells are not present in the 2-D model but may be a point of interaction for *C. jejuni* with the intestine, this may partially limit the usefulness of the classical 2D model.

An EVOC model has successfully been established to study the interaction of enteropathogenic and enterohaemorrhagic *E. coli* (EPEC and EHEC) with human intestinal biopsies (Hicks *et al.*, 1996). The model has been used to highlight similarities as well as differences between the interactions of EHEC with a classical 2-D IEC monolayer and with the biopsies. For example, pedestal

formation and the formation of attaching and effacing lesions occur in both models (Hicks *et al.*, 1998). However, certain differences between the two models regarding the molecular basis of the interactions have also been discovered (Schuller *et al.*, 2007). For *C. jejuni*, four studies have utilised human intestinal biopsies. As the tissue is relatively difficult to obtain, these remain the only studies to date.

1.5 Aims

Although some progress has been made over the past decade, the knowledge about how *C. jejuni* causes disease in humans still lags behind that for other pathogenic enteric bacteria. The aims of this research project were to further develop and assess two alternative *in vitro* models to study the interactions of this important bacterial pathogen with the host.

1.5.1 The *Ex Vivo* Organ Culture (EVOC) model

One of the drawbacks of IEC-bacterial co-culture is the absence of cell types other than the IECs. Co-culturing whole human intestinal biopsies with *C. jejuni* will address this, as these tissues contain all cell types present in the human intestinal lining, including DCs, macrophages and neutrophils. The innate immune response mounted by the tissue in response to *ex vivo* infection with *C. jejuni* will be studied. Furthermore, the effect of loss of bacterial surface structures on the response will be addressed by using different *C. jejuni* mutants.

1.5.2 The Vertical Diffusion Chamber (VDC) model

Another major drawback of IEC co-culturing with *C. jejuni* is the aerobic conditions that such experiments are performed under. The VDC model aims to overcome this problem by co-culturing *C. jejuni* with IECs under microaerobic conditions, thus more closely mimicking the conditions encountered by *C. jejuni* *in vivo* in the human intestine. Initially, survival of the IECs under microaerobic conditions will be analysed using different methodologies. Subsequently, the effect of microaerobic co-culturing of *C. jejuni* with the IECs will be analysed, both with regards to the numbers of interacting and invading bacteria as well as with regards to the host innate immune response mounted after microaerobic co-culturing. Finally, different *C. jejuni* mutants will be tested using the VDC model.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Bacterial strains

Campylobacter jejuni strains used in this study are listed in Table 2.1. The two *C. jejuni* wild-type strains used were 11168H, a hypermotile derivative of the original sequenced strain NCTC11168 (Jones *et al.*, 2004, Karlyshev *et al.*, 2001), and 81-176, a strain isolated from a milk-borne outbreak in the USA (Korlath *et al.*, 1985). The defined *C. jejuni* 11168H mutants used in the study were obtained from Nevada Naz (*ciaB*), the LSHTM *Campylobacter jejuni* mutant library (*flaA*) or Nick Dorrell (*rpoN*). All three mutants were constructed by insertion of a Kanamycin resistance cassette into the Open Reading Frame at the nucleotide position specified in Table 2.1. (Karlyshev *et al.*, 2001)

Table 2.1: *C. jejuni* strains used in this study.

Strain/Mutant	Origin/Reference	Features
11168H wild-type strain	Karlyshev <i>et al.</i> , 2001, Jones <i>et al.</i> , 2004	Hypermotile derivative of the original sequenced strain NCTC11168
11168H strain expressing enhanced Green Fluorescent Protein (eGFP)	Karlyshev and Wren, 2005	11168H strain modified to express eGFP
81-176 wild-type strain	Korlath <i>et al.</i> , 1985	Wild-type laboratory passaged strain
11168H <i>ciaB</i> mutant	Obtained from Nevada Naz, LSHTM	Kan ^R cassette inserted into the gene encoding CiaB at nucleotide 103
11168H <i>flaA</i> mutant	Obtained from LSHTM mutant library	Kan ^R cassette inserted into the gene encoding the major flagellin subunit protein FlaA at nucleotide 163
11168H <i>rpoN</i> mutant	Obtained from Nick Dorrell, LSHTM	Kan ^R cassette inserted into the gene encoding the alternative sigma factor σ^{54} (RpoN) at nucleotides 127, removing nucleotides 128 to 1036

Complemented 11168H <i>rpoN</i> mutant	This study	Functional copy of the 11168H <i>rpoN</i> gene inserted into the <i>Cj0223</i> pseudogene locus (nucleotides 205297–207475 inclusive)
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For cloning and plasmid propagation, library efficiency *Escherichia coli* DH5α chemically competent cells (Invitrogen, Paisley, UK) were used.

2.2 Bacterial growth media and culturing conditions

All bacterial growth media were prepared using Milli-RO (Reverse osmosis) pure water (Millipore, Billerica, USA).

C. jejuni were routinely cultured on Blood Agar (BA) plates supplemented with *Campylobacter* selective supplement (Skirrow) (Oxoid, Basingstoke, UK) and 7% (v/v) horse blood (TCS Biosciences, Botolph Claydon, UK). BA was prepared by dissolving 21 g of Columbia Agar Base (Oxoid) in 465 ml of H₂O and autoclaving at 121°C for 15 min in a steam steriliser. To prepare agar plates, the agar base was melted in a microwave and cooled to approximately 50°C. 35 ml of horse blood and one vial of the selective supplement were added to the cooled agar and 25 ml of the mixture was poured into each sterile petri dish (Fisher Scientific, Leicestershire, UK). When required, kanamycin (Sigma. Gillingham, UK) (final concentration 50 µg/ml) and chloramphenicol (Sigma) (final concentration 10 µg/ml) were added to the cooled agar immediately before pouring.

E. coli were routinely grown on Luria Bertani Agar (LBA) plates. LBA was prepared by dissolving 18.5 g of LB agar base (Oxoid) in 500 ml H₂O and autoclaving at 121°C for 15 min. To prepare agar plates, the agar base was melted in a microwave, cooled to approximately 50°C and 25 ml poured into each sterile petri dish. When required, ampicillin (Sigma) (final concentration 50 µg/ml), kanamycin (Sigma) (final concentration 50 µg/ml) or chloramphenicol (Sigma) (final concentration 30 µg/ml) were added to the cooled agar immediately before pouring.

C. jejuni motility medium was prepared by dissolving 5.6 g of Brucella broth base (Oxoid) and 0.8 g bacto-agar (Oxoid) (final agar concentration 0.4% (w/v)) in 200 ml H₂O and autoclaving at 121°C for 15 min. To prepare motility plates, the agar

was melted in a microwave, cooled to approximately 50°C and 20 ml poured into each sterile petri dish.

C. jejuni cultures were grown in Brucella broth. Brucella broth was prepared by dissolving 5.6 g of Brucella broth base in 200 ml H₂O and autoclaving at 121°C for 15 min.

E. coli cultures were routinely grown in LB broth. LB broth was prepared by dissolving 5 g of LB broth base (Oxoid) in 200 ml H₂O and autoclaving at 121°C for 15 min.

C. jejuni were routinely grown at 37°C in a Variable Atmosphere Incubator (VAIN) (Don Whitley Scientific, Shipley, UK) filled with a mixture of 85% N₂, 10% CO₂ and 5% O₂. *C. jejuni* cultures were grown in vented 75 cm² tissue culture flasks (Corning NY, USA) at 75 revolutions per minute (RPM) on an SSM-1 orbital shaker (Stuart/Bibby Scientific, Stone, UK) inside the VAIN.

E. coli strains were routinely grown on LBA plates at 37°C in a MIR microbiological incubator (Sanyo, Loughborough, UK). *E. coli* cultures were grown at 37°C in a shaking incubator (Gallenkamp/Sanyo, Loughborough, UK) at 200 RPM.

2.3 Preparation of frozen bacterial stocks

To prepare frozen stocks of *C. jejuni*, bacteria were grown for 24 h on a BA plate. Bacteria were harvested with a sterile 10 µl plastic loop (VWR, Lutterworth, UK), resuspended in Brucella broth supplemented with 10% (v/v) foetal calf serum (FCS) (Sigma) and 10% (v/v) Glycerol (Sigma), aliquotted into 1.5 ml Eppendorf tubes (Eppendorf, Histon, UK) and snap-frozen by immersion into an ethanol-dry-ice mix for 1 min. Frozen stocks were stored at -80°C in a New Brunswick Scientific Innova ultra-low freezer (Eppendorf)

To prepare frozen stocks of *E. coli*, the bacteria were grown overnight in LB broth, 800 µl of bacterial culture mixed with 200 µl of sterile Glycerol in a Cryovial (NUNC Roskilde, Denmark) and the vial frozen down directly to -80°C in a New Brunswick Scientific Innova ultra-low freezer.

2.4 Resuscitation of bacteria from frozen stocks

To resuscitate *C. jejuni* from frozen stocks, an Eppendorf tube containing the desired bacterial strain was slowly thawed on ice. 25 µl of stock suspension were streaked onto a BA plate with the aim of obtaining single colonies. After 24 h of growth, one or more CFUs were re-streaked onto a fresh BA plate; this was termed passage number 1.

Resuscitation of *E. coli* from frozen stocks was performed as above but using LBA instead of BA plates.

2.5 Passaging of *C. jejuni* strains

C. jejuni strains were routinely passaged onto a fresh BA plate every 72-96 h up to a maximum passage number of 10. Passages were routinely checked for contamination by streaking in a fashion aimed at obtaining single CFUs and the morphology of the CFUs examined, as extended passage can lead to the development of a mucoidy colony phenotype. If a mucoidy phenotype was observed, the line was discontinued, and fresh bacteria resuscitated from the stocks as described in section 2.4.

E. coli strains were not routinely passaged. Instead, plates were kept at 4°C for up to 4 weeks after resuscitation and single colonies inoculated into fresh LB broth when new liquid cultures were required. After 4 weeks of storage, a fresh plate was prepared as described in section 2.4.

2.6 Preparation of a *C. jejuni* suspension for assays

To prepare suspensions of *C. jejuni* for assays, bacteria were grown for 24 h on a BA plate, collected from the plate using a sterile 10 µl loop and resuspended in the desired medium.

2.7 Quantification of bacteria

Two methods were used to quantify the number of bacteria in a suspension:-

2.7.1 Quantification by optical density

For rapid quantification of bacteria in a suspension, the optical density (OD) at a wavelength of 600 nm was determined. Bacteria were suspended in the required

medium as described in section 2.6 and diluted 1:10 in fresh medium in a cuvette (10 mm path length, Kartell, Noviglio, Italy). The spectrophotometer (WPA, Cambridge, UK) was zeroed with a sample of pure medium and the OD₆₀₀ of the bacterial suspension determined. This method allows for rapid quantification of different bacterial suspensions such as for inoculation of a co-culture assay with different strains/mutants with approximately the same number of bacteria. However, this method is only an approximation and requires the OD₆₀₀/CFU ratio to be similar for every strain and mutant. This was assessed using the CFU count method of bacterial quantification.

2.7.2 Quantification by CFU count

For absolute quantification of bacterial numbers in a suspension, such as for determination of the OD₆₀₀/CFU ratio for different *C. jejuni* strains and mutants, a bacterial suspension was adjusted to an OD₆₀₀ of 1. The suspension was serially diluted 1:10 in phosphate buffered saline (PBS) (Sigma) up to a dilution factor of 10⁶ and 100 µl aliquots of each dilution plated in triplicate on BA plates and incubated for 72 h. For dilutions with 20-200 CFUs per plate, the number of CFUs was counted and used to calculate total CFUs in the initial suspension. Using this method, a concentration of 1-2 x 10⁹ *C. jejuni* / ml was determined for a suspension of OD₆₀₀ = 1. This was confirmed to be the case for both wild-type strains as well as all mutants used in this study.

2.8 Mammalian cell culture

2.8.1 Human intestinal epithelial cell lines

Two human intestinal epithelial cell (IEC) lines were used in this study: Caco-2 (ATCC® number HTB-37™) and T84 (ATCC® number CCL-248™). Both lines are derived from a colorectal adenocarcinoma and exhibit characteristics of a polarised epithelium such as tight junctions and desmosomes when grown to confluence. Both lines were routinely cultured at 37°C in a MCO15-AC humidified tissue culture incubator (Sanyo) in air enriched with 5% CO₂.

2.8.2 Cell culture media

Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% (v/v) heat-deactivated FCS, 1% (v/v) MEM non-essential amino acids (Sigma), 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma). T84 cells were grown in 1:1 DMEM/Ham's F12 medium containing GlutaMAX™ and supplemented with 10% (v/v) heat inactivated FCS, 100 units/ml penicillin and 100 µg/ml streptomycin. These two media are referred to as complete growth medium in this study.

2.8.3 Resuscitation of IECs from frozen stocks

Frozen stocks of both IEC lines were stored in the vapour phase of a Statebourne Biosystems²⁴ liquid nitrogen storage tank (Statebourne, Washington Tyne & Wear, UK). To resuscitate the IECs, a vial was thawed at 37°C and the contents added to 10 ml of the appropriate complete growth medium in a 25 cm² tissue culture flask (Corning). After 24 h, the medium was removed, the cells washed once with sterile PBS (Sigma) and 10 ml of fresh complete growth medium added. Once the cells reached 80-90% confluence as judged by eye using an inverted microscope (Leica, Wetzlar, Germany), the cells were detached by immersion in Trypsin-EDTA solution (Sigma) for 5-10 min and seeded into a new 75 cm² tissue culture flask (Corning) containing 25 ml of fresh complete growth medium.

2.8.4 Passaging of IECs

IECs were routinely subcultured (split) when 80-90% confluence was reached. The cells were briefly washed with 10 ml of sterile PBS and then with 1.5 ml of Trypsin-EDTA solution. 4 ml of fresh Trypsin-EDTA was added to the cells and incubated for 5-10 min at 37°C in the tissue culture incubator to detach the cells. The Trypsin was then deactivated by addition of 46 ml of fresh complete growth medium containing 10% (v/v) FCS. The cell suspension was centrifuged at 200 x g for 10 min at 4°C. The cell pellet was resuspended in fresh culture medium by gentle pipetting and one third (T84) or one eighth (Caco-2) of the suspension transferred to a fresh 75 cm² flask containing 25 ml of complete growth medium (Split ratio 1:3 or 1:8 respectively).

2.8.5 Enumeration of IECs using a haemocytometer

100 µl of cell suspension was mixed with 800 µl of complete growth medium and 100 µl of Trypan Blue dye (Sigma). Approximately 10 µl was loaded into both chambers of a haemocytometer (Weber Scientific, Teddington, UK) and the average number of cells per square determined using an inverted microscope. The cell density was calculated as described in Figure 2.1.

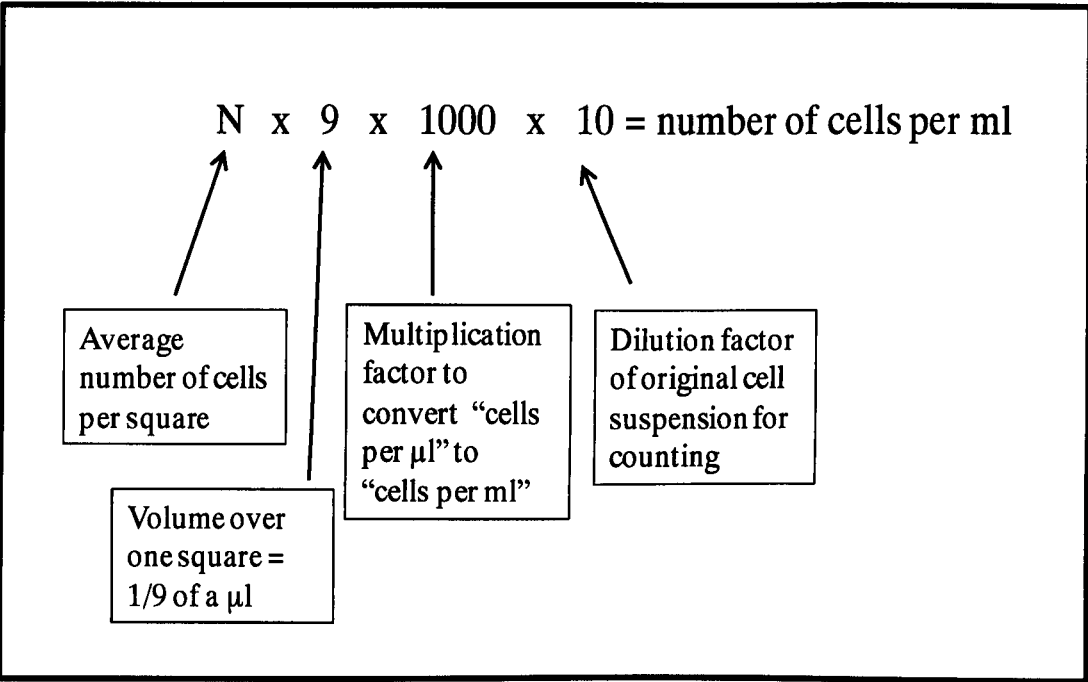


Figure 2.1: Formula used to calculate the cell density of a suspension when N cells were counted using the haemocytometer.

Suspensions of the required density were obtained by diluting the original suspension with fresh complete growth medium.

2.8.6 Preparation of frozen IEC stocks

IECs were grown in 75 cm² tissue culture flasks, detached with Trypsin-EDTA, washed and counted as described in section 2.8.4 and 2.8.5. The cells were pelleted again and resuspended in complete growth medium without antibiotics but supplemented with 5% (v/v) dimethyl sulfoxide (DMSO) as a cryoprotectant at a density of 2-4 x 10⁶ cells/ml. 1 ml aliquots of the suspension were added to cryovials

(NUNC) and gradually cooled down to -80°C overnight (O/N) using a Mr Frosty™ freezing box (NUNC) in a New Brunswick Scientific Innova ultra-low freezer. The vials were then transferred to the vapour phase of a Statebourne Biosystems²⁴ liquid nitrogen storage tank.

2.8.7 Culturing of IECs on Snapwell™ filters for co-culturing experiments in a Vertical Diffusion Chamber (VDC)

For subsequent IEC-bacterium co-culture studies in a VDC, IECs were seeded onto permeable polycarbonate Snapwell™ filters (Pore size 0.4 µm) (Corning) suspended from hanging supports at a density of 4×10^5 cells/filter. The IECs were grown on the Snapwell™ filters in a MCO15-AC humidified tissue culture incubator for a minimum of 21 days, during which the medium was changed every three days.

2.8.8 Measurement of the Trans-Epithelial Electrical Resistance (TEER) of IECs growing on Snapwell™ filters

The TEER of IECs growing on Snapwell™ filters was measured using a Millicell ERS-2 resistance meter using chopstick electrodes (Millipore).

2.9 Recombinant DNA techniques

Ultra-pure Milli-Q H₂O (Millipore) was used for all molecular biology techniques.

2.9.1 Isolation of *C. jejuni* genomic DNA

C. jejuni genomic DNA was isolated from bacteria grown for 24 h on BA plates using a Gentra Puregene Bacterial Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

2.9.2 Polymerase chain reaction (PCR)

PCR was routinely performed using *GoTaq* DNA polymerase (Promega, Southampton, UK). If high fidelity amplification was desired, either for large amplicons or for recombinant protein expression, Accuprime *Pfx* supermix (Invitrogen) was used. The PCR mixtures were set up as follows:

10 X PCR Buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl)	5 μ l
50 mM MgCl ₂	1.5 μ l
10 mM dNTP mix (each dNTP at 10 mM)	1 μ l
Oligonucleotide primers (10 pmol)	2 μ l
0.5 units DNA polymerase	0.2 μ l
Template DNA (10 pg to 200 ng)	1-5 μ l
H ₂ O	to 50 μ l

The reaction mixture was scaled up for reactions aimed at obtaining large yields of PCR product for downstream applications and scaled down to 25 μ l for screening PCRs.

PCR reactions were routinely performed on a Tetrad-2 thermocycler (BioRad, Hemel Hempstead, UK). A representative PCR program using *GoTaq* DNA polymerase is shown below:

95°C for 10 min initial denaturation	- 1 cycle
95°C denaturation for 30 s	} - 35 cycles
55°C annealing for 30 s	
72°C extension for 1 min/ kb	
72°C for 10 min	- 1 cycle

The annealing temperature was adjusted depending on the characteristics of the primer pair. If *Pfx* supermix was used, the extension temperature was changed to 68°C and the extension time adjusted to 1 min/500 bp.

2.9.3 Purification of PCR products and purification of DNA after enzymatic reactions

DNA fragments were purified after PCR and after digestion with restriction endonucleases using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

2.9.4 DNA digestion using restriction endonucleases

Restriction endonucleases were purchased from New England Biolabs (NEB, Hitchin, UK). Restriction digests were routinely set up as follows:

10 X Reaction buffer (NEB)	5 µl
Restriction endonuclease	5 U
DNA	0.5 µg to 5 µg
H ₂ O	to 50 µl

Digests were routinely performed at 37°C for 1 h. Double digests were performed as required if the two enzymes shared a common reaction buffer. DNA was purified after digestion as described in section 2.9.3.

2.9.5 Antarctic phosphatase treatment of digested DNA vectors

To prevent self-ligation of digested vectors, 5' phosphate groups were removed by treatment with Antarctic phosphatase (NEB) at 37°C for 15 min according to the manufacturer's instructions.

2.9.6 Ligation of DNA fragments

T4 DNA ligase was purchased from NEB. Insert-vector ligation reactions were as follows:

10 X Reaction buffer	1µl
T4 DNA ligase (400,000 cohesive end units/ml)	1 µl
Vector backbone DNA	2 µl
Insert DNA	6 µl
H ₂ O	to 10 µl

Insert to vector ratios of 3:1 were used. Ligations were mixed by gentle pipetting and incubated at 4°C O/N.

2.9.7 Transformation of *E. coli* with ligation products/plasmids

E. coli DH5α cells were transformed with ligation products or plasmids using a heat shock protocol. Briefly, 100 µl of chemically competent *E. coli* DH5α cells were thawed on ice and gently mixed with 1 µl of plasmid or 5 µl of ligation product. The mixture was incubated on ice for another 30 min followed by heat shocking at 42°C for 45 seconds. The cells were cooled on ice for 2 min before 900 µl of SOC broth (Invitrogen) was added and the cells recovered by incubation for 1.5 h at 37°C with agitation at 200 RPM. The bacteria were harvested by centrifugation at 13,000 x RPM for 2 min, the supernatant removed by decanting, the cells resuspended in the residual supernatant, plated on selective agar plates and incubated O/N at 37°C.

2.9.8 Isolation of plasmid DNA from *E. coli* Strains

Plasmid DNA was isolated from overnight cultures of *E. coli* using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

2.9.9 Agarose gel electrophoresis (AGE) of DNA fragments

50 X concentrated tris-acetic acid EDTA buffer (TAE) was prepared as follows:

Trizma-base (Sigma)	242 g
0.5 M EDTA pH 8.0 (Sigma)	100 ml
Glacial Acetic Acid (Sigma)	57.2 ml
H ₂ O	to 1 l

To prepare a 1% (w/v) agarose gel for DNA electrophoresis, 1 g of agarose powder (Bioline, London, UK) was added to 100 ml of 1 X TAE buffer. The agarose was dissolved by microwaving the suspension at a low setting to avoid evaporation and superheating. Once the agarose was dissolved, the suspension was cooled to 50°C, Ethidium Bromide (stock 10 mg/ml) (Promega) that had been stored in the dark at 4°C was added at a final concentration of 0.5 µg/ml, the gel poured into an electrophoresis tray (BioRad), the comb (BioRad) inserted and the gel left to set at

room temperature. The DNA fragments were mixed with 6 X concentrated loading buffer (Bioline) in a ratio of 6:1, loaded into the wells, separated by electrophoresis at 100 V for 45 min in an AGE tank (BioRad) and the separated fragments visualised by UV-illumination of the EtBr-DNA complex in a Gene Genius Gel imager (UVP, Cambridge, UK).

2.9.10 Transformation of *C. jejuni* with plasmid DNA

Electrocompetent *C. jejuni* for transformation with plasmid DNA by electroporation were prepared as follows. *C. jejuni* were grown on BA plates for 24 h. The bacteria were harvested with a sterile loop and thoroughly resuspended in 1 ml of ice cold EBF solution (10% (v/v) glycerol, 10% (w/v) sucrose in H₂O). The bacteria were pelleted by centrifugation at 13,000 x RPM for 10 min and resuspended in 1 ml of ice cold EBF solution. This step was repeated twice. The bacteria were then resuspended in 200 µl of ice cold EBF and 50 µl aliquots placed into sterile microcentrifuge tubes. 5 µl of plasmid DNA was added to the bacterial suspension, mixed by gently flicking the tube and incubated for 15 min on ice. The mixture was transferred to a pre-cooled electroporation cuvette (BioRad) (2 mm diameter) and placed into a Gene Pulser XCell electroporator (BioRad). The suspension was electroporated with the following settings (2.5 kV, 25 µF, 200 Ω), after which 100 µl of prewarmed SOC broth (Invitrogen) was added and thoroughly mixed. The suspension was plated onto non-selective BA plates and incubated microaerobically for 24 h. The resultant lawn was collected with a sterile loop, streaked onto a BA plate containing the appropriate antibiotic and incubated microaerobically for up to 72 h. Colonies were restreaked onto fresh selective BA plates and checked by PCR to verify the homologous recombination of the desired construct.

2.9.11 Complementation of the *C. jejuni* *rpoN* mutation

The complementation vector described in (Hitchen *et al.*, 2010) was used to complement the *rpoN* mutation in the 11168H *rpoN* mutant. The vector had been engineered to carry a 2179 bp fragment corresponding to nucleotides 205297–207475 of the *C. jejuni* 11168H genome. This stretch of nucleotides is annotated as a pseudogene, *Cj0223* (Gundogdu *et al.*, 2007). A chloramphenicol resistance cassette lacking a transcriptional terminator, followed by a multiple cloning site (MCS)

comprised of recognition sites for the restriction endonucleases *NcoI* and *NheI* was cloned into the centre of the 2179 bp fragment. The *C. jejuni* gene to be complemented can therefore be directionally cloned into the MCS and will be expressed from the constitutively active Cam^R promoter.

A forward primer was designed to anneal 30 bp upstream of the ATG start codon of the *rpoN* gene in order to include the ribosomal binding site (RBS), as no RBS is present on the complementation vector (Figure 2.2). An *NcoI* restriction enzyme recognition site was included at the 5' end of the primer for directional cloning of the PCR product into the complementation vector.

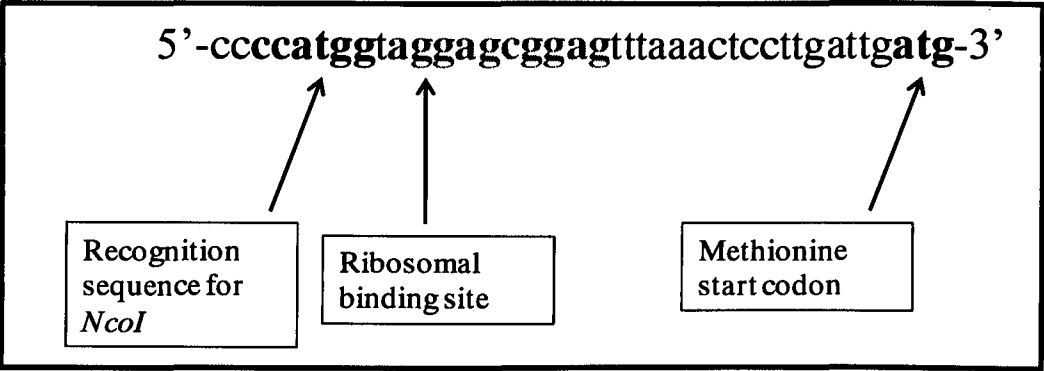


Figure 2.2: Forward primer used to amplify the *rpoN* gene from genomic DNA of *C. jejuni* 11168H wild-type strain.

A reverse primer was designed to anneal at the 3' end of the *rpoN* gene .An *NheI* restriction enzyme recognition site was included at the 5' end of the primer for directional cloning of the PCR product into the complementation vector (Figure 2.3).

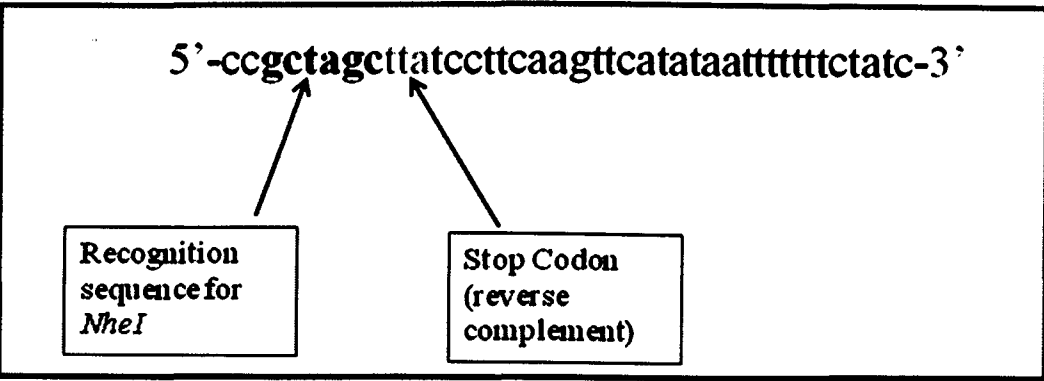


Figure 2.3: Reverse primer used to amplify the *rpoN* gene from genomic DNA of *C. jejuni* 11168H wild-type strain.

The *rpoN* gene was amplified by PCR from genomic DNA isolated from the *C. jejuni* 11168H wild-type strain using the two primers shown above. The PCR product was digested with *NcoI* and *NheI*, purified, ligated into the *NcoI/NheI* site of the complementation vector and transformed into chemically competent *E. coli* as described in sections 2.9.4-5 and 2.9.6-7. Successful cloning of the PCR product was confirmed by purifying plasmid *prpoNcomp* from *E. coli* and performing restriction analysis on the plasmid DNA using *NcoI* and *NheI*. Analysis of the restriction products by AGE demonstrated the release of a DNA fragment of the correct size from both plasmids, but not from the original empty vector used for cloning, confirming the successful cloning of a DNA fragment of the correct size for *rpoN* into the complementation vector.

2.10 Host-pathogen interaction methods

2.10.1 Assembly of the Vertical Diffusion Chamber (VDC)

Prior to assembly, the two compartments of the VDC (Harvard Apparatus, Kent, UK) were sterilised by immersion into Chlorox (Sigma) solution for a minimum of 3 h, followed by three washes with Milli-RO water. A Snapwell™ filter carrying a monolayer of Caco-2 cells differentiated for at least 21 days was removed from a culture plate, washed three times with PBS and inserted between the two compartments of the VDC. The apical compartment was filled first to avoid removal of the IECs from the Snapwell™ filter due to liquid pressure from the basolateral compartment. The apical compartment was filled with either DMEM supplemented with 1% (v/v) FCS and 1% (v/v) non-essential amino acid solution when using Caco-2 IECs, 1:1 DMEM/Ham's F12 medium containing GlutaMAX™ supplemented with 1% (v/v) heat inactivated FCS when using T84 IECs or with 4 ml of Brucella broth. The basolateral compartment was filled with 4 ml of DMEM supplemented with 1% (v/v) FCS and 1% (v/v) non-essential amino acid solution when using Caco-2 IECs, 1:1 DMEM/Ham's F12 medium containing GlutaMAX™ supplemented with 1% (v/v) heat inactivated FCS when using T84 IECs. For co-culturing experiments, *C. jejuni* were harvested from a BA plate grown for 24 h, adjusted to approximately 1×10^8 CFU/ml in either DMEM or Brucella broth as described in section 2.6 and 4 ml added into the apical compartment. For aerobic co-

incubation, the VDC was maintained at 37°C in 5% CO₂ and 95% air in the humidified tissue culture incubator. For microaerobic co-incubation, the VDC was maintained under microaerobic conditions (85% N₂, 10% CO₂, and 5% O₂) in a VAIN. During microaerobic co-culturing, a gas mixture of 95% O₂, 5% CO₂ was perfused through the basolateral compartment using a 12-channel gas manifold (Harvard Apparatus) at a flow rate of 1 bubble every 2 to 3 seconds, while the apical compartment was left open to the microaerobic atmosphere of the VAIN.

2.10.2 Measurement of the TEER of IEC monolayers in the VDC

The TEER of an IEC monolayer on a Snapwell™ filter in a VDC was measured using a DVC-1000 voltage clamp (World Precision Instruments, Stevenage, UK). This was performed by placing two voltage sensing AgCl electrodes close to the cell monolayer on each side of the Snapwell™ filter, passing a current through two further electrodes placed at the two distal ends of the VDC and reading the voltage necessary to keep the current flowing. Resistance was calculated according to Ohm's law (Resistance equals Voltage divided by Current ($R = V/I$)), and multiplied by the surface area of the monolayers (1.12 cm²), to give the TEER in Ω cm².

2.10.3 Enumeration of bacteria interacting (adherent and invaded) with the IECs

At specific time points, the apical and basolateral supernatants were removed from the VDC and stored at -80°C for subsequent analysis of the host immune response (see section 2.10.5). Alternatively, for microarray analysis of the gene expression of *C. jejuni* after co-culturing with the IECs in the VDC, the apical supernatant was removed, mixed 1:2 with RNeasy Protect (Qiagen) and stored at -80°C. The Snapwell™ filter was removed from the VDC, washed three times with sterile PBS and placed into a 6-well tissue culture dish (Corning). The Caco-2 cells were lysed by addition of 0.5% (v/v) Triton X-100 (Sigma) in PBS for 20 min at 37°C. The lysates were serially diluted in PBS, plated on BA and incubated for 72 h. CFU counts were determined and the number of bacteria interacting with the IECs calculated.

2.10.4 Enumeration of intracellular bacteria

Enumeration of intracellular bacteria was essentially performed as above, with one modification. Before lysis with Triton X-100, the Caco-2 cells were incubated with 150 µg/ml Gentamicin (Sigma) in DMEM for 2 h at 37°C. This level of Gentamicin has been shown to kill *C. jejuni* whilst not permeating epithelial cells. This step therefore kills extracellular, adherent bacteria and allows for the enumeration of the intracellular bacteria present after co-incubation.

2.10.5 Analysis of the host innate immune response

Co-incubation supernatants were probed for the presence of chemokines, cytokines and defensins with the appropriate human cytokine/defensin ELISA Development Kit (Peprotech, London, UK), according to the manufacturer's instructions. Briefly, a 96-well microtitreplate (NUNC) was coated overnight with anti-human cytokine/defensin capture antibody at room temperature. The plate was washed three times with 0.1% (v/v) Tween 20 (Sigma) in PBS (PBS-Tween) and blocked for 2 h with 1% (w/v) Bovine Serum Albumin (BSA) (Sigma) in PBS. Standards containing known concentrations of chemokine/cytokine/defensins were prepared, loaded with the samples in duplicate and incubated at room temperature for 2 h. The plate was washed three times with PBS-Tween and the wells incubated with biotinylated anti-human chemokine/cytokine/defensin detection antibody at room temperature for 1 h (concentration according to the manufacturer's instructions). The plate was washed 3 times with PBS-Tween and the wells incubated with Horseradish Peroxidase (HRP) conjugated avidin at room temperature for 30 min (concentration according to the manufacturer's instructions). The plate was then washed three times with PBS-Tween and 100 µl of 2,2'-azino-bis-(3-benzthiazoline-6-sulfonic acid) (ABTS) substrate solution (Sigma) was added. The absorbance of each well at 405 nm was measured on a Dynex MRX II 96-well plate reader (Dynex, Worthing, UK). The data was processed with Revelation software (Revelation, Ealing, UK) and the chemokine/cytokine/defensin concentration of each sample calculated using a standard curve generated from the standard calibration samples.

2.10.6 Immunofluorescence analysis of Caco-2 cells on Snapwell™ filters

After disassembly of the VDC post-infection, the Caco-2 cells were fixed with 2% (w/v) paraformaldehyde (Sigma) for 1 h at 4°C. The fixed cells were permeabilised with 0.1% (v/v) Triton X-100 in PBS for 20 min at room temperature and blocked with 1% (w/v) BSA in PBS for 1 h at room temperature. The filter was then excised from the carrier and placed in a 12-well dish. For actin staining, the cells were incubated with Alexa Fluor 555 conjugated phalloidin (Invitrogen) (stock diluted 1:1000 in PBS) for 1 h in the dark. For staining of occludin, the cells were incubated with mouse anti-occludin primary antibody (stock diluted 1:100 in PBS) (Invitrogen) for 1 h at room temperature followed by an Alexa Fluor 488 conjugated goat anti-mouse (Invitrogen) (stock diluted 1:200 in PBS) for 1 h at room temperature in the dark. Stained filters were mounted in Vectashield mounting medium containing 4',6 diamidino-2-phenylindole (DAPI, final concentration 1.5 µg/ml) (Vector Laboratories, Peterborough, UK) on a coverslip (Fisher) and examined with a Zeiss LSM510 Confocal microscope (Carl Zeiss AG, Jena, Germany).

2.10.7 Fluorescent dextran diffusion assay

IECs on Snapwell™ filters were washed three times with sterile PBS, placed back into the hanging support and 500 µl of 100 µM FITC-labelled dextran (Sigma) with an average molecular weight of 4 kilodaltons (kDa) in Ringer's solution (115 mM NaCl, 1 mM KCl, 1 mM CaCl₂ in H₂O) added to the apical side of the monolayer and incubated for 3 h at room temperature, with the basolateral side of the monolayer immersed in Ringers solution. The amount of fluorescently labelled dextran on the basolateral side of the monolayer was determined post-incubation by removal of the basolateral solution and measurement of the fluorescence intensity at 488 nm using a Gemini XPS Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, USA).

2.10.8 Preparation of media for *ex vivo* organ culture

EVOC medium was prepared by adding 0.95 g powdered NCTC-135 media (Sigma) to 100 ml H₂O. The pH was adjusted to 7.4 by adding 0.22 g sodium bicarbonate (Sigma). Finally, 100 ml of DMEM (Sigma) and 20 ml FCS (Sigma) were added and

the medium filter sterilised using a 0.22 μm filter (Millipore) to make up complete EVOC medium.

2.11 Bacteriological assays

2.11.1 Swimming motility assay

To assess the motility of a *C. jejuni* strain, the ability of the bacteria to swim through 0.4% (w/v) soft agar plates was determined. Motility plates were prepared as described in section 2.2. Two different methods for inoculating the bacteria into the agar were used. For a quick check of motility, a culture growing on a BA plate was touched with a sterile loop, stabbed into the motility plate and the motility monitored over 72 h. For a more quantitative comparison between strains, a bacterial suspension of 1 OD₆₀₀ was prepared in sterile PBS as described in section 2.6, then 1 μl stabbed into the motility plate using a pipette and the motility monitored by measuring the diameter of the bacterial halo every 24 h over a period of 72 h.

2.11.2 Autoagglutination assay

To assess the autoagglutination properties of a *C. jejuni* strain or mutant, 2 ml of a suspension at an OD₆₀₀ of 1 was prepared in sterile PBS as described in section 2.6. The suspension was incubated in a 5 ml plastic tube (Fisher) at 37°C under microaerobic conditions for 24 h and the OD₆₀₀ of the top 1 ml of the suspension was determined as described in section 2.6. If the bacteria tested had autoagglutinated, the OD would be substantially lower, as most of the bacteria would have pelleted to the bottom of the tube.

2.12 Microarray analysis

2.12.1 Total RNA isolation

Total bacterial RNA was isolated using the RNEasy kit (Qiagen) according to the manufacturer's instructions from apical supernatants of VDC co-culturing experiments that had been stored in RNAProtect.

2.12.2 Microarray experimental design, template labelling and hybridisation

Gene expression profiling of *C. jejuni* 11168H wild-type strain co-cultured with Caco-2 IECs for 6 and 24 h in the VDC under either aerobic or microaerobic conditions was performed using an indirect comparison method or type 2 experimental design (Kamal *et al.*, 2007). In this experimental design, replicate test sets of Cy5-labelled *C. jejuni* 11168H wild-type strain total RNA samples were combined with a common reference sample (Cy3-labelled *C. jejuni* 11168H wild-type strain genomic DNA). The microarrays used in this study were whole genome *C. jejuni* NCTC11168 arrays printed on Ultragaps glass slides (Corning), produced by the BµG@S Group (www.bugs.sghms.ac.uk).

50 ml of pre-hybridisation solution was prepared as stated below and placed into a Coplin jar (Fisher). Reagents were added in the following order:-

Sodium Dodecyl Sulphate (SDS) 20% (v/v) (Sigma)	250 µl
H ₂ O	- 36.0 ml
20 X Saline Sodium Citrate (SSC) (Sigma)	8.75 ml
Bovine Serum Albumin (BSA) (100 mg/ml in H ₂ O)	5 ml

The pre-hybridisation solution was incubated at 65°C for 1 h before beginning the experiment.

2.12.2.1 Cy3-labelling of control *C. jejuni* 11168H wild-type strain genomic DNA

To label the control genomic DNA, the following reaction was set up:-

Genomic DNA	2 µg
Random primers (3 µg/µl) (Invitrogen)	- 1 µl
H ₂ O	to 41.5 µl

Reactions were heated at 95°C for 5 min followed by snap cooling on ice and brief pulse centrifugation. The following reagents were added to the reactions:-

10 X REact 2 buffer (Invitrogen)	5 µl
dNTPs (5 mM dATP, dGTP dTTP, 2 mM dCTP) (Invitrogen)	1 µl
25 nM Cy3-labelled dCTP (GE Healthcare, Chalfont St. Giles, UK)	1.5 µl
Klenow fragment (10 U/µl) (Invitrogen)	1 µl

The reaction was incubated at 37°C for 90 min.

2.12.2.2 Cy5-labelling of test *C. jejuni* 11168H wild-type total RNA

To label the test RNA, the following reaction was set up.

Total RNA	-	2.5 ng
Random primers (3 µg/µl) (Invitrogen)	-	1 µl

Reactions were heated at 95°C for 5 minutes followed by snap cooling on ice and brief pulse centrifugation. Test reactions were prepared, adding reagents in the following order:-

5 X first strand buffer (Invitrogen)	5 µl
DTT (100 mM) (Sigma)	2.5 µl
dNTPs (5 mM dATP, dGTP dTTP, 2 mM dCTP) (Invitrogen)	2.3 µl
25 nM Cy5-labelled dCTP (GE Healthcare)	1.7 µl
Reverse transcriptase (Superscript II 200 U/µl) (Invitrogen)	2.5 µl

The Test Cy5-labelled reaction was incubated at 25°C for 10 min, followed by incubation at 42°C for 90 min. 20 min prior to completion of this incubation, microarray slide(s) were placed into the pre-hybridisation solution in the Coplin jar and incubated at 65°C.

2.12.2.3 Combination and purification of the labelled test and control samples

Both control and test reactions were combined in a 1.5 ml microcentrifuge tube. The reaction was purified using a MinElute PCR purification kit (Qiagen) according to the manufacturer's instructions. The cDNA was eluted from the column by adding 14 μ l H₂O to the spin column. Samples were incubated on ice. Pre-hybridized microarray slides were rinsed in H₂O for 1 minute using a trough and holder (Fisher). Slides were then rinsed in 100% isopropanol for 1 minute. Microarray slides were dried by placing into 50 ml Falcon tubes and centrifuging at 1500 x RPM for 5 min. The Hybridisation solution was prepared as follows in a 1.5 ml microcentrifuge tube:-

Eluted sample	14 μ l
H ₂ O	26 μ l
20 X SSC	12 μ l
2% (v/v) SDS	9 μ l

This hybridisation solution was heated at 95°C for 2 min, allowed to cool to room temperature and briefly centrifuged.

A microarray slide was placed in a humidified hybridisation cassette (Telechem International, Sunnyvale, USA) and covered with a LifterSlip™ (Erie Scientific, Portsmouth, USA) glass coverslip (22 mm x 25 mm) over the array section. The hybridisation solution was carefully pipetted under the LifterSlip™. The microarray chamber was sealed and incubated in a water bath at 65°C overnight without shaking. Wash A solution was also prepared and incubated at 65°C overnight along with troughs and holder.

Wash A

20 X SSC	20 ml
20% (v/v) SDS	1 ml
H ₂ O	379 ml

Wash A was dispensed into a single trough. Prior to commencing the wash protocol, Wash B solution was prepared as follows;

Wash B

20 X SSC	2.4 ml
H ₂ O	797.6 ml

Wash B was dispensed into two troughs.

The microarray slides were removed from the cassettes, placed into the holder in Wash A and gently agitated for 5 min. The microarray slides were transferred to a clean rack and rinsed with gentle agitation in Wash B for 2 min. This was followed by another 2 min in the second Wash B trough. The microarray slides were placed into 50 ml Falcon tubes (Fisher) and spun at 1500 x RPM for 5 min to dry the slides, with the labels facing away from the tube lid to ensure any liquid was centrifuged away from the printed array area.

2.12.3 Data analysis

The microarray slides were scanned with an Affymetrix 418 array scanner (MWG Biotech, Acton, UK) according to the manufacturer's instructions. Signal and local background intensity readings for each spot were quantified using ImaGene software v8.0 (BioDiscovery, El Segundo, USA). Quantified data were analysed using GeneSpring GX software v7.2 (Agilent, Santa Clara, USA). Expression analysis was performed using a DNA versus RNA experimental set-up. To determine which genes were differentially expressed under the microaerobic test conditions, a *P*-value of < 0.05 was used for testing whether the normalised expression of each gene statistically differed from 1.0 by ANOVA (Analysis Of Variance) using a Benjamini & Hochberg False Discovery Rate as the Multiple Testing Correction within the GeneSpring software. As many of the fold changes between the gene expression levels under the two experimental conditions were small, this correction method was used as it is the least stringent method and therefore least likely to call a gene non-significant when in fact it is.

Chapter 3

Development of an *ex vivo* organ culture model to study *C. jejuni* interactions with human intestinal biopsies

3.1 Introduction

One advantage of animal models for infectious diseases over most *in vitro* models is the complexity of the *in vivo* system, despite the fact that such complexity may complicate the monitoring of the interaction between pathogen and host. In the case of the intestinal pathogen *C. jejuni*, it is assumed that the bacteria interact with numerous host cells types such as epithelial cells, DCs sampling the intestinal lumen and also with macrophages and other cells of the innate and adaptive immune system. The commonly used *in vitro* models generally utilise only one cell type, which therefore do not take into account the complexity of the interactions between the pathogen and the host or the interactions between host cells after encountering the pathogen, resulting in a simplification of the interactions occurring *in vivo*. This may in turn lead to an incomplete understanding of the host-pathogen interactions involved in pathogenesis. Small animal models can be used to overcome the simplicity of single cell type *in vitro* models, as they offer a more complex basis for investigating host-pathogen interactions. However no robust, reproducible and routinely usable small animal model has been established to date for *C. jejuni* infection. So far, most advances in the understanding of *C. jejuni* pathogenesis have been achieved using *in vitro* models with either immortalised cell lines or primary tissue.

An *ex vivo* organ culture (EVOC) model, where human primary tissue is co-cultured with a pathogen, is one way of overcoming the absence of an animal model whilst not compromising the complexity of the tissue. The major drawback with the EVOC model is that the timeframe of such experiments is limited due to the problems with viability of the tissue over a prolonged period of time *ex vivo*. An EVOC model has successfully been utilised to study the interaction of enteroaggregative, enteropathogenic and enterohaemorrhagic *Escherichia coli* with human intestinal tissue (Hicks *et al.*, 1996, Hicks *et al.*, 1998, Mousnier *et al.*, 2008). An example of the advantage of using primary human intestinal tissue was a study that demonstrated differences between Tir phosphorylation and Nck/N-WASP recruitment in an EVOC model for enterohaemorrhagic and enteropathogenic *E. coli* compared to those observed in cell culture models (Schuller *et al.*, 2007). For *C. jejuni*, the first report of an EVOC model demonstrated activation of ERK, JNK and

p38 MAPK kinases in an EVOC model (MacCallum *et al.*, 2005a). A second study demonstrated the secretion of IL-8 from *C. jejuni* infected human intestinal tissue (MacCallum *et al.*, 2006). Initial microscopical studies of the interaction between *C. jejuni* and human intestinal tissue *ex vivo* have demonstrated micro-colonies of bacteria interacting with the tissue and with other bacteria, preferentially via the flagella (Grant *et al.*, 2006). This observation has been confirmed recently with the demonstration that *C. jejuni* form microcolonies on *ex vivo* infected human ileal tissue (Haddock *et al.*, 2010).

The aim of this study was to expand the use of the EVOC model for studying the host innate immune response to *C. jejuni* infection using different *C. jejuni* wild-type strains as well as defined isogenic mutants.

3.2 Setting up the EVOC model

Human intestinal biopsies were co-cultured with *C. jejuni* as described previously (Hicks *et al.*, 1996). In brief, histologically normal mucosal samples from terminal ileum and colon were obtained courtesy of Dr Keith J Lindley from paediatric patients undergoing routine endoscopic examination at Great Ormond Street Hospital for Children. Institutional ethical approval (ICH/Great Ormond Street Hospital for Children Research Ethics Committee: REC: 06/Q0508/26-05/07/2006 and LSHTM Ethics Committee: EC:5016-19/09/2006) was obtained in advance and parental consent obtained at the time of the procedure. The patients' age varied between 2 and 16 years, with a median of 11 years of age. Routinely, around 2-4 biopsies from the colon were obtained. If the patient was undergoing upper endoscopy, around 1-3 additional ileal biopsies were received. An experimental day usually encompassed working with biopsies from between 1 and 3 patients. The tissue was placed into EVOC medium (see section 2.10.8) immediately after removal of the tissue from the patient and transported to the lab. The biopsies were microscopically examined for integrity such as presence of villi for ileal samples or intact crypts for colonic samples. If satisfactory, biopsies were mounted with the mucosal side upwards on sterile foam blocks in a 12 well plate (Figure 3.1). EVOC medium was added to the wells to a level that allowed partial covering of the biopsies. Preliminary studies had demonstrated that tissue stayed intact under these conditions for at least 12 hours (Matthias Zilbauer, PhD thesis, University of

London, 2007). *C. jejuni* were harvested from a 24 h plate into sterile PBS and adjusted to approximately 1×10^9 CFU/ml. 20 μ l of this suspension was inoculated onto the tissue and incubated in a tissue culture incubator at 37°C with agitation on a rocker (30 motions per min). The EVOC medium was changed after 4 hours and another 20 μ l of the same bacterial suspension inoculated onto the biopsies. 8 hours after the initial set up, the medium (containing secreted proteins from time point 4 h to time point 8 h) was removed and stored at -80°C for subsequent analysis.

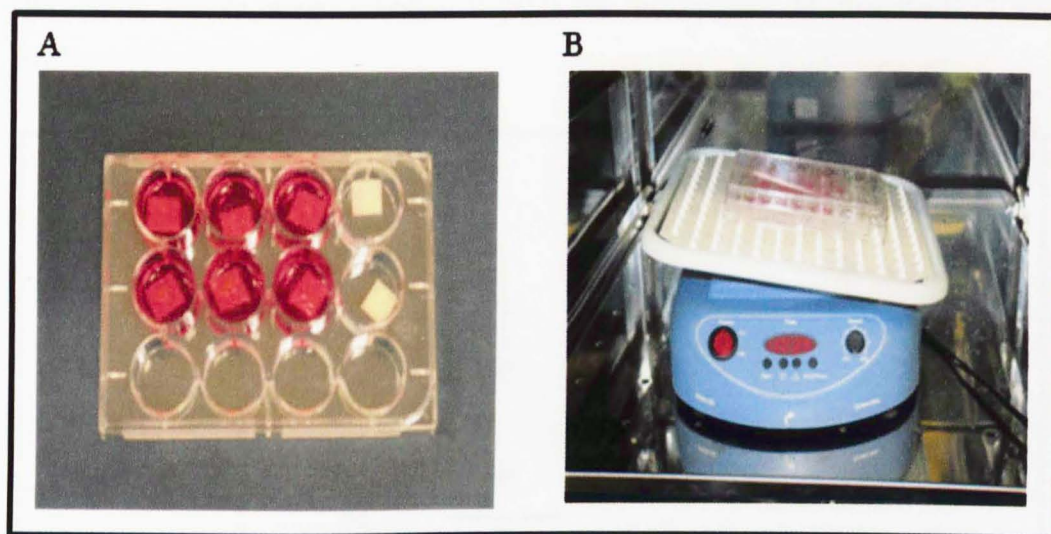


Figure 3.1: Microscopically intact biopsies were mounted mucosal side up on sterile foam blocks and covered with *ex vivo* organ culture medium. Biopsies were inoculated with either sterile PBS as a negative control or with 20 μ l of a suspension of a *C. jejuni* wild-type strain or mutant in PBS containing approximately 1×10^9 CFU/ml and incubated on a rocker at 37°C in a tissue culture incubator containing air enriched with 5% CO₂.

3.3 Co-incubation of human intestinal tissue with *C. jejuni* 11168H wild-type strains *ex vivo* does not lead to increased secretion of Interleukin 8 (IL-8)

Previous studies have shown that the secretion of the proinflammatory chemokine IL-8 by adult human intestinal tissue was increased in response to *ex vivo* infection with *C. jejuni* wild-type strains NCTC11168 and L115 (MacCallum *et al.*, 2006). Human intestinal biopsies from the ileum and the colon were either mock infected with PBS or infected with *C. jejuni* 11168H wild-type strain for 8 h *ex vivo*. The

supernatants were analysed by ELISA for the presence of IL-8 (Figure 3.2). No difference in IL-8 levels in the supernatants of infected and uninfected tissue was observed, in contrast to the previously reported study. The basal levels of IL-8 secretion from uninfected tissue was similar for the ileal samples, 500 pg/ml in the previously published study (MacCallum *et al.*, 2006) and 380 pg/ml in this study. However, the basal levels of IL-8 secreted from the uninfected colonic samples differed markedly, approximately 2000 pg/ml in the previously published study and 360 pg/ml in this study. Possible reasons for this finding are discussed in section 3.11.

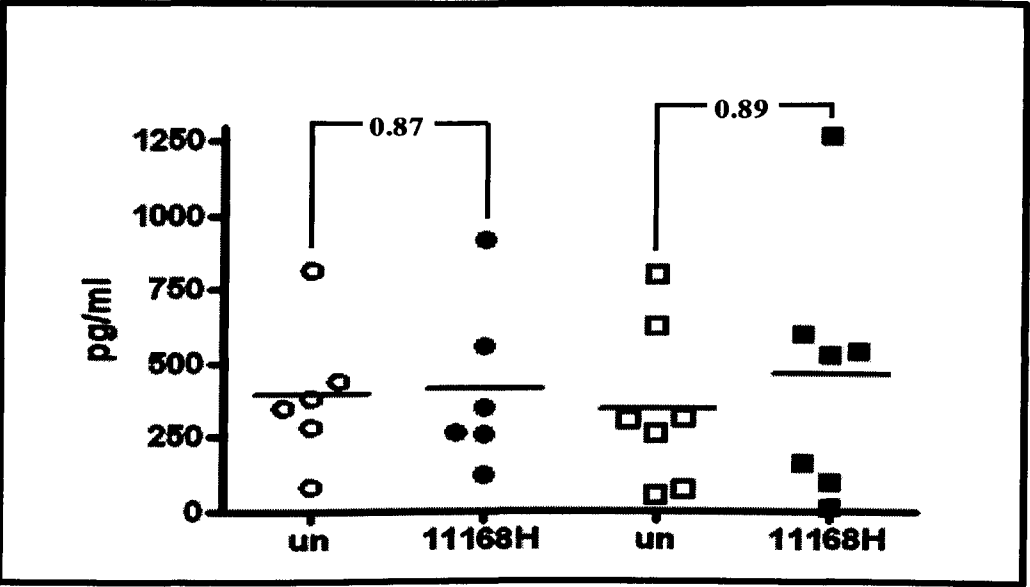


Figure 3.2: Human intestinal biopsies from the ileum (circles, N = 6) and the colon (squares, N = 7) were either mock infected with PBS (open symbols) or infected with *C. jejuni* 11168H wild-type strain (black symbols) for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of IL-8.

3.4 Co-incubation of human intestinal tissue with *C. jejuni* 81-176 wild-type strains *ex vivo* does not lead to increased secretion of IL-8

The previously published study demonstrated different levels of IL-8 secretion by human intestinal tissue in response to infection with two different *C. jejuni* wild-type strains (MacCallum *et al.*, 2006), with wild-type strain NCTC11168 inducing lower

levels of IL-8 secretion than strain L115. To investigate the possibility that the *C. jejuni* 11168H wild-type strain only induces a minimal IL-8 response in this EVOC model and that a different *C. jejuni* wild-type strain might induce higher levels of IL-8 secretion, human intestinal biopsies from the ileum and the colon were either mock infected with PBS or infected with *C. jejuni* 81-176 wild-type strain for 8 h *ex vivo*. The supernatants were analysed by ELISA for the presence of IL-8.

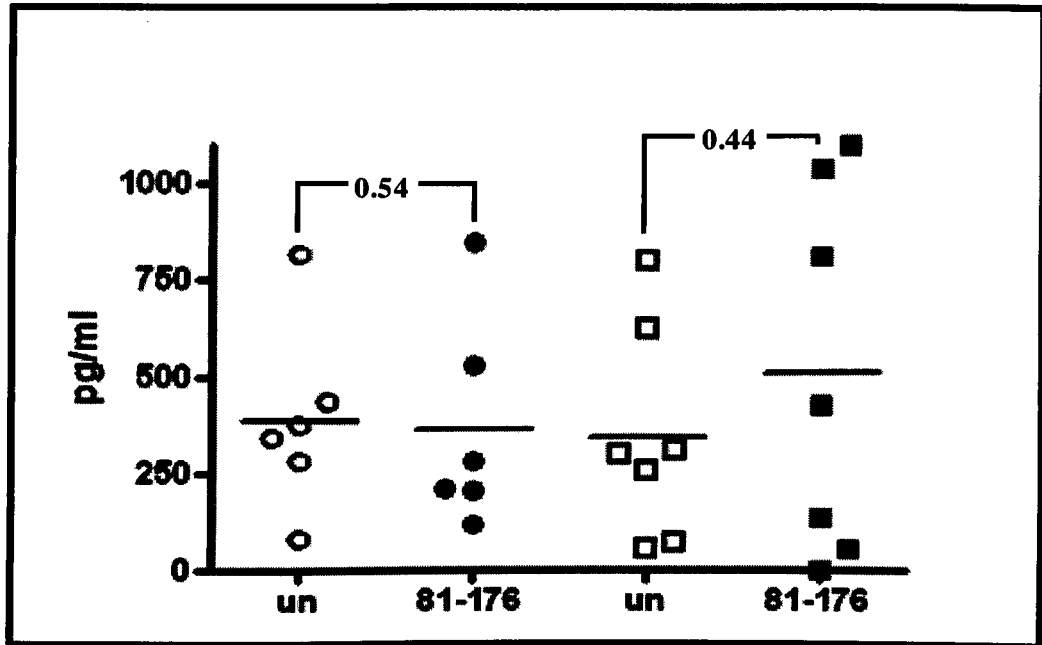


Figure 3.3: Human intestinal biopsies from the ileum (circles, N = 6) and the colon (squares, N = 7) were either mock infected with PBS (open symbols) or infected with *C. jejuni* 81-176 wild-type strain (black symbols) for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of IL-8.

As with infection with the *C. jejuni* 11168H wild-type strain, no difference was detected between the levels of IL-8 secreted from uninfected biopsies compared to biopsies infected *ex vivo* with the *C. jejuni* 81-176 wild-type strain (Figure 3.3).

3.5 Co-incubation of human intestinal tissue with *C. jejuni* 11168H or 81-176 wild-type strains *ex vivo* results in increased secretion of human beta defensins (hBDs)

hBDs are antimicrobial peptides involved in innate immune defences against pathogenic bacteria. Infection with *C. jejuni* leads to increased transcription,

translation and secretion of human beta defensins 2 and 3 (hBD-2 and hBD-3) from Caco-2 and HT-29 human IECs (Zilbauer *et al.*, 2005). This *in vitro* data suggested an important role for hBDs in responding to *C. jejuni* infection. As no increased secretion of IL-8 had been detected from either colonic or ileal human tissue in response to infection with either *C. jejuni* 11168H or 81-176 wild-type strains (Figure 3.2 and Figure 3.3), the effect of infection with the same two wild-type strains on the secretion of hBD-2 and hBD-3 was analysed. To determine whether a hBD response was elicited in *ex vivo* infected human primary tissue, the co-culture supernatants were probed for the presence of hBD-2 and hBD-3 by ELISA following *ex vivo* infection with the two different *C. jejuni* wild-type strains 11168H and 81-176 for 8 h.

3.5.1 hBD-2

hBD-2 is a cysteine-rich cationic low molecular weight antimicrobial peptide, which has been shown to be produced by a number of epithelial cells and exhibits potent antimicrobial activity against Gram-negative bacteria (Schroder *et al.*, 1999). hBD-2 showed potent bactericidal activity against *C. jejuni* 11168H wild-type strain *in vitro*, with > 99% of all bacteria in an initial suspension of 1×10^5 CFU killed within 30 min of exposure (Zilbauer *et al.*, 2005).

Human intestinal biopsies from the ileum and the colon were either mock infected with PBS or infected with *C. jejuni* 11168H or 81-176 wild-type strains for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of hBD-2 (Figure 3.4).

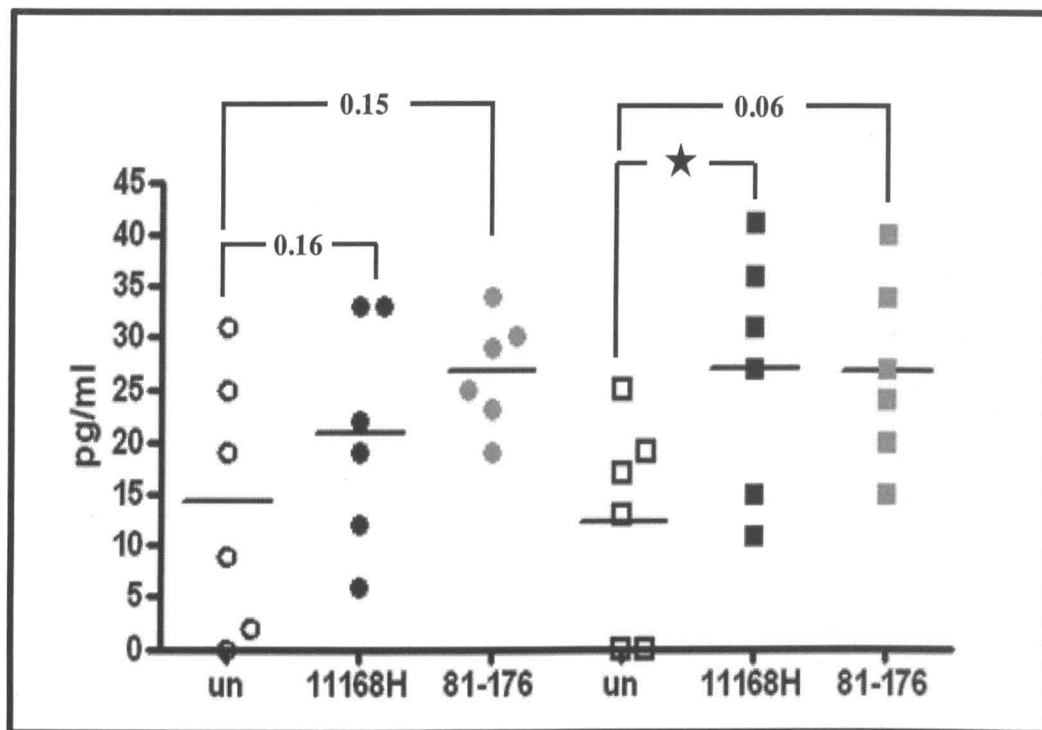


Figure 3.4: Human intestinal biopsies from the ileum (circles, N = 6) and the colon (squares, N = 6) were either mock infected with PBS (open symbols) or infected with *C. jejuni* 11168H (black symbols) or 81-176 (grey symbols) wild-type strains for 8 h *ex vivo*. The supernatants were probed for the presence of hBD-2. * = $p < 0.05$

Basal levels of hBD-2 were detected in the supernatants of uninfected biopsies from the ileum and the colon. (Figure 3.4, open circles and squares, respectively). Increased levels of hBD-2 were detected in supernatants of both ileal and colonic biopsies infected with *C. jejuni* 11168H wild-type strain (Figure 3.4, black circles and squares) with the numbers reaching statistical significance for colonic tissue. The supernatants of biopsies infected with *C. jejuni* 81-176 wild-type strain also contained increased levels of hBD-2 compared to the uninfected samples (grey circles and squares), however the differences did not reach statistical significance. Taken together, the results demonstrate that infection of human intestinal tissue with *C. jejuni* 11168H and 81-176 wild-type strains *ex vivo* leads to induction of hBD-2, supporting previous evidence indicating a role for the hBD-2 in the defence against the pathogen. The data also indicates that a host response is induced by *C. jejuni* with this EVOC model, in contrast to the data obtained for IL-8.

3.5.2 hBD-3

hBD-3 is a small, cationic antimicrobial peptide that has been shown to be active against Gram-negative as well as Gram-positive bacteria (Schneider *et al.*, 2005). Similar to hBD-2, hBD-3 expression has been demonstrated to be upregulated in IECs *in vitro* and to exhibit bactericidal effects against *C. jejuni* 11168H wild-type strain *in vitro* similar to hBD-2, with > 99% of all bacteria in an initial suspension of 1×10^5 CFU killed within 30 min of exposure (Zilbauer *et al.*, 2005). Human intestinal biopsies from the ileum and the colon were either mock infected with PBS or infected with *C. jejuni* 11168H or 81-176 wild-type strains for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of hBD-3 (Figure 3.5)

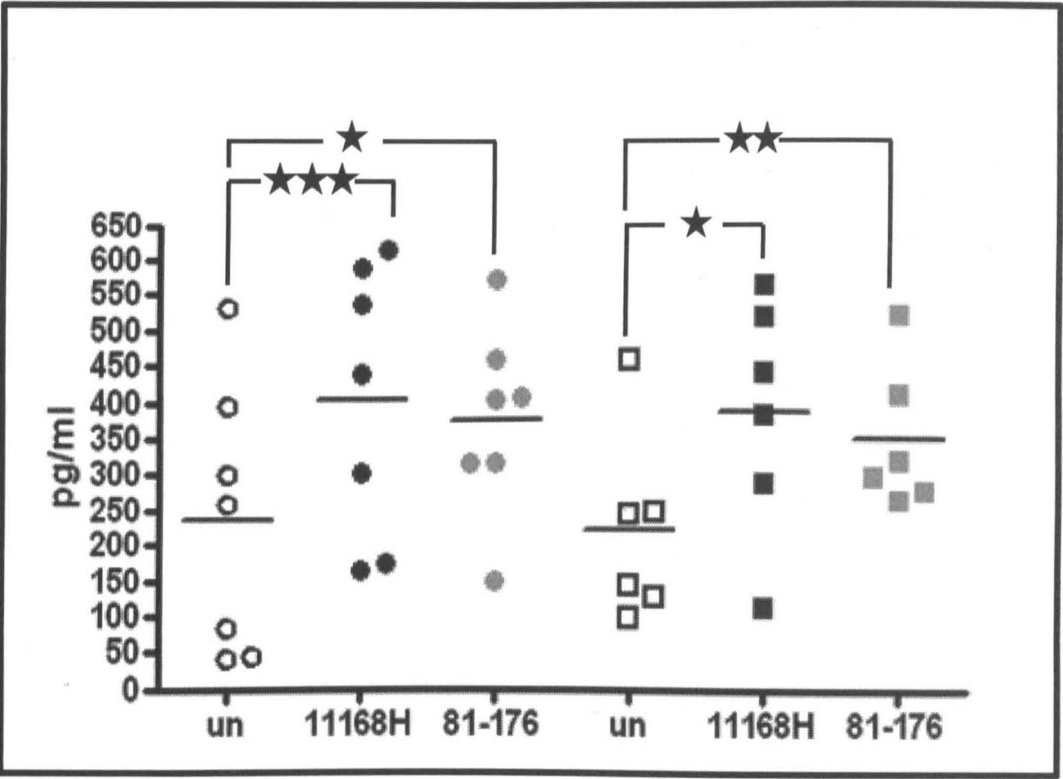


Figure 3.5: Human intestinal biopsies from the ileum (circles, N = 7) and the colon (squares, N = 6) were either mock infected with PBS (open symbols) or infected with *C. jejuni* 11168H (black symbols) or 81-176 (grey symbols) wild-type strains for 8 h *ex vivo*. The supernatants were probed for the presence of hBD-3. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Basal levels of hBD-3 were detected in the supernatants of uninfected tissue (Figure 3.5, open circles and squares). Compared to hBD-2, the levels of hBD-3 detected in

uninfected samples were considerably higher, with an average of 240 pg/ml and 230 pg/ml for ileal and colonic tissue respectively compared to approximately 14 pg/ml and 13 pg/ml for hBD-2. As with hBD-2, increased levels of hBD-3 were detected in both the ileal and the colonic supernatants from biopsies infected with either *C. jejuni* 11168H (Figure 3.5, black circles and squares) or 81-176 wild-type strain (Figure 3.5, grey circles and squares). Furthermore, all differences between infected and uninfected biopsy supernatants reached statistical significance.

Taken together, these results demonstrate that hBD-2 and hBD-3 are induced from human intestinal biopsies in response to infection with *C. jejuni* wild-type strains *ex vivo*. This supports the previously published *in vitro* data (Zilbauer *et al.*, 2005) and suggests that both defensins play an important part in the host innate immune defence against *C. jejuni*. This is the first time that secretion of hBD-2 and hBD-3 has been demonstrated in response to challenge of human intestinal tissue *ex vivo* with *C. jejuni*. Furthermore, as no significant difference was detected between the innate immune response induced by the two *C. jejuni* wild-type strains tested and in light of the limited availability of primary tissue, combined with the necessity for at least 5 replicate data points per experiment, the decision was taken to use only the *C. jejuni* 11168H wild-type strain in future experiments.

3.6 Co-incubation of human intestinal tissue with *C. jejuni* 11168H wild-type strain *ex vivo* results in increased secretion of IL-1 β

IL-1 β is a pro-inflammatory cytokine that is secreted upon recognition of pathogenic microorganisms. IL-1 β has been shown to induce the release of other pro-inflammatory cytokines such as tumor necrosis factor (TNF) and IL-6. IL-1 β has also been implicated in inducing a Th17 bias in the cellular adaptive immune response (Netea *et al.*, 2010). IL-1 β release from human DCs and macrophages in response to *C. jejuni* has previously been demonstrated (Hu *et al.*, 2006, Siegesmund *et al.*, 2004).

Human intestinal biopsies from the ileum and the colon were either mock infected with PBS or infected with *C. jejuni* 11168H wild-type strain for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of IL-1 β (Figure 3.6).

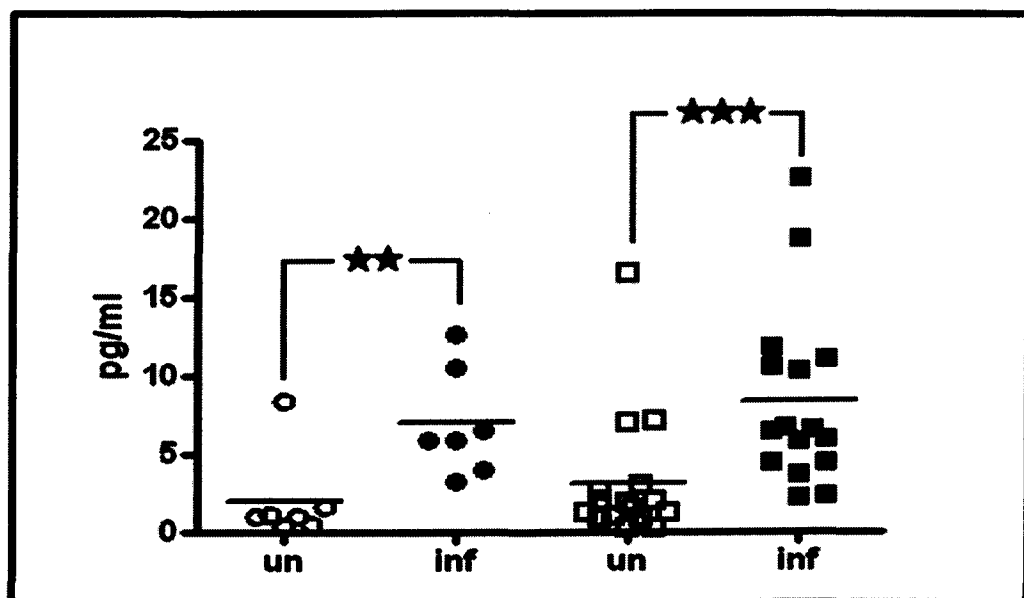


Figure 3.6: Human intestinal biopsies from the ileum (circles, N = 7) and the colon (squares, N = 16) were either mock infected with PBS (open symbols) or infected with *C. jejuni* 11168H wild-type strain (black symbols) for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of IL-1 β . ** = $p < 0.01$, *** $p < 0.001$

Significantly more IL-1 β was detected in the supernatants of *C. jejuni* 11168H wild-type strain infected biopsies compared to the uninfected controls (Figure 3.6). The secreted quantities were relatively low, however as only macrophages and DCs have been implicated in IL-1 β secretion, this was not surprising. No striking difference was observed between the ileal and the colonic samples. This data suggests a role for IL-1 β in the host defence against *C. jejuni* and supports previous data. Furthermore, this is the first time that secretion of IL-1 β has been demonstrated in response to challenge of human intestinal tissue *ex vivo* with *C. jejuni*

3.7 Co-incubation of human intestinal tissue with *C. jejuni* 11168H wild-type strain *ex vivo* results in increased secretion of IL-6

IL-6 is involved in maintaining the intestinal epithelium, but is also a pro-inflammatory cytokine secreted by epithelial cells as well as macrophages and T-cells in response to pathogens (Jones, 2005). IL-6 has been shown to be produced by DCs as well as intestinal epithelial cells in response to *C. jejuni* infection *in vitro* (Friis *et al.*, 2009, Rathinam *et al.*, 2008).

Human intestinal biopsies from the ileum and the colon were either mock infected with PBS or infected with *C. jejuni* 11168H wild-type strain for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of IL-6 (Figure 3.7).

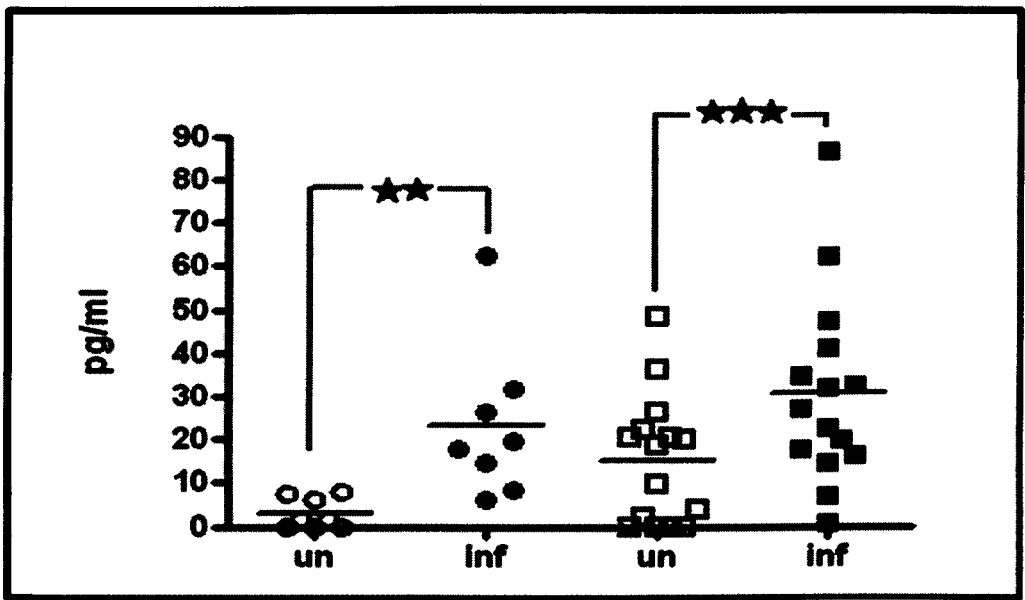


Figure 3.7: Human intestinal biopsies from the ileum (circles, N =8) and the colon (squares, N =15) were either mock infected with PBS (open symbols) or infected with *C. jejuni* 11168H wild-type strain (black symbols) for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of IL-6. ** = $p < 0.01$, *** $p < 0.001$.

Significantly higher levels of IL-6 were detected in the supernatants of biopsies that had been infected with *C. jejuni* 11168H wild-type strain compared with the uninfected controls (Figure 3.7). The supernatants of infected colonic samples contained on average more IL-6 than the ileal samples (31 pg/ml compared to 23 pg/ml), however this was also the case for the uninfected samples.

This data confirms previous evidence from cell culture assays (Friis *et al.*, 2009) and DCs (Rathinam *et al.*, 2008) and highlights a role for IL-6 in the host innate immune response to *C. jejuni*. Furthermore, this is the first time that secretion of IL-6 has been demonstrated in response to challenge of human intestinal tissue *ex vivo* with *C. jejuni*

3.8 Co-incubation of human intestinal tissue with *C. jejuni* 11168H wild-type strain *ex vivo* results in increased secretion of IL-12 and IL-23, suggesting a possible involvement of a Th17 adaptive immune response in combating *C. jejuni* infection

Data obtained with collaborators at the ICH demonstrated that *in vitro* co-culturing of *C. jejuni* 11168H wild-type strain with human blood derived DCs led to secretion of IL-1 β and IL-6, supporting the findings reported in the previous sections. Additionally, IL-12 and IL-23, two hallmarks of a Th17 pro-inflammatory immune response, were detected in the supernatants. Furthermore, incubation of the co-culturing supernatants with human T-cells led to the production of Interferon Gamma (IFN- γ) and IL-17, suggesting that *C. jejuni* modulates the Th17 type adaptive immune response (Edwards *et al.*, 2010).

As DCs are most likely present in the biopsies, supernatants of *C. jejuni* infected intestinal biopsies were analysed for the presence of IL-12 and IL-23.

3.8.1 IL-12

The IL-12 family of cytokines are important players in the regulation of T-cell responses, and are produced by monocytes, macrophages and DCs in response to infection (Gee *et al.*, 2009). IL-12 has been demonstrated to be secreted by both human and murine DCs in response to *C. jejuni* challenge *in vitro* (Hu *et al.*, 2006). Human intestinal biopsies from the ileum and the colon were either mock infected with PBS or infected with *C. jejuni* 11168H wild-type strain for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of IL-12 (Figure 3.8)

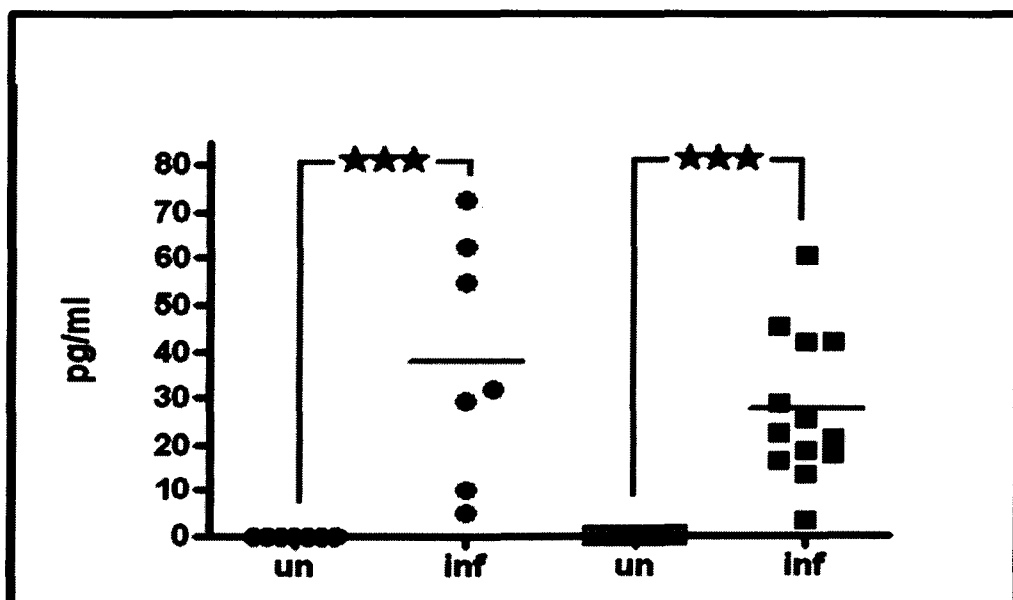


Figure 3.8: Human intestinal biopsies from the ileum (circles, N = 7) and the colon (squares, N = 13) were either mock infected with PBS (open symbols) or infected with *C. jejuni* 11168H wild-type strain (black symbols) for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of IL-12. *** $p < 0.001$.

Significantly higher levels of IL-12 were detected in the supernatants of tissue that had been incubated with *C. jejuni* 11168H wild-type strain compared to the uninfected controls (Figure 3.8). In contrast to what had been observed previously for IL-8, hBD-2, hBD-3, IL-1 β and IL-6, no basal levels of IL-12 secretion were detected in the uninfected controls.

3.8.2 IL-23

IL-23 is a recent addition to the ever increasing number of signalling molecules that regulate the host adaptive immune system (Monteleone *et al.*, 2009). Similar to IL-12, IL-23 has been shown to act as a link between innate and adaptive immunity. Together with IL-12, IL-23 has been postulated to lead to the differentiation of naïve T cells into a novel class of T-helper cells, termed Th17, for the ability to secrete the cytokine IL-17. By stimulating the production of pro-inflammatory cytokines, the IL-17 secreting T cells are then involved in mediating inflammation (Mills, 2008).

Human intestinal biopsies from the ileum and the colon were either mock infected with PBS or infected with *C. jejuni* 11168H wild-type strain for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of IL-23 (Figure 3.9).

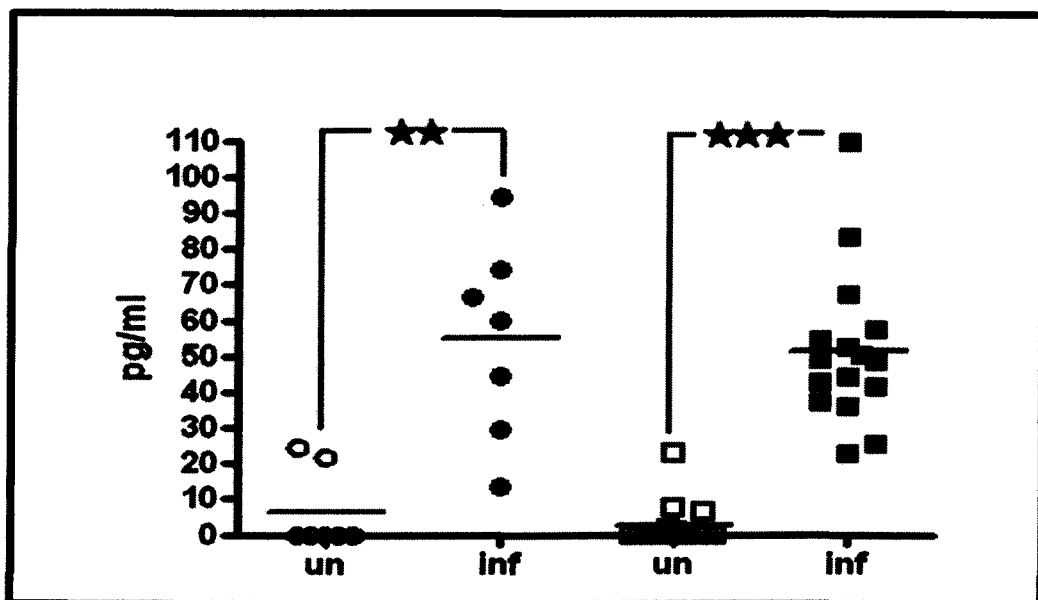


Figure 3.9: Human intestinal biopsies from the ileum (circles, N = 7) and the colon (squares, N = 16) were either mock infected with PBS (open symbols) or infected with *C. jejuni* 11168H wild-type strain (black symbols) for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of IL-23. ** = $p < 0.01$, *** $p < 0.001$.

Significantly higher levels of IL-23 were detected in the supernatants of tissue that had been incubated with *C. jejuni* 11168H wild-type strain compared to the uninfected controls (Figure 3.9).

This supports recent data obtained by *in vitro* culturing of human blood-derived DCs with *C. jejuni* where secretion of IL-1 β , IL-6, IL-12 and IL-23 was detected after the DCs were exposed to *C. jejuni*. Furthermore, the relative abundance of the four cytokines (IL-1 β < IL-6 < IL-12 < IL-23) that was observed for the infected biopsies also held true for the DCs (Edwards *et al.*, 2010). Whether this finding is a result of the different relative numbers of cytokine producing cells remains unclear.

3.9 Co-incubation of human intestinal tissue with *C. jejuni* 11168H wild-type strain *ex vivo* results in increased secretion of IFN- γ and IL-17

The presence of both IL-12 and IL-23 in the supernatants of both infected DCs as well as infected human intestinal biopsies suggests that *C. jejuni* infection triggers the IL-12/IL-23 immune axis. This axis has been proposed to further modulate

downstream T-cell responses, in particular the recently identified novel acute neutrophilic Th17 response, characterised by the differentiation of IL-17-producing Th17 cells (van de Veerdonk *et al.*, 2009). When naïve T cells were cultured *in vitro* with supernatants from DC-*C. jejuni* co-cultures, both IFN- γ and IL-17 were detected, suggesting that *C. jejuni* triggered both a Th1 and a Th17 response (Edwards *et al.*, 2010).

Human intestinal biopsies from the ileum and the colon were either mock infected with PBS or infected with *C. jejuni* 11168H wild-type strain for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of IFN- γ (Figure 3.10).

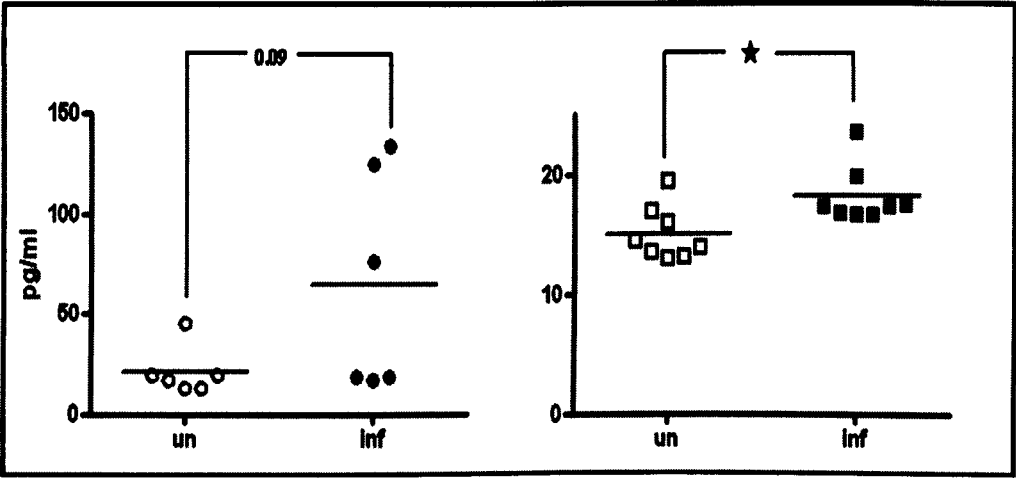


Figure 3.10: Human intestinal biopsies from the ileum (circles, N = 6) and the colon (squares, N = 8) were either mock infected with PBS (open symbols) or infected with *C. jejuni* 11168H wild-type strain (black symbols) for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of IFN- γ . * = $p < 0.05$.

Higher levels of IFN- γ were detected in the supernatants of *C. jejuni* infected tissue compared to the uninfected controls (Figure 3.10). However, the data only reached statistical significance for the colonic samples. Interestingly, the absolute values of detected IFN- γ were approximately 3-fold higher in the ileal samples than in the colonic samples (65 pg/ml for the ileum compared to 18 pg/ml for the colon), a trend that was not observed with any of the other cytokines tested. As the source of the cytokines detected in the EVOC model cannot be easily traced back to the cell type involved in secretion, it is unclear whether this difference is the result of a different relative abundance of IFN- γ secreting cells.

Data obtained with the ICH collaborators demonstrated increased levels of IL-17 in supernatants of *ex vivo* infected human intestinal biopsies (Figure 3.11).

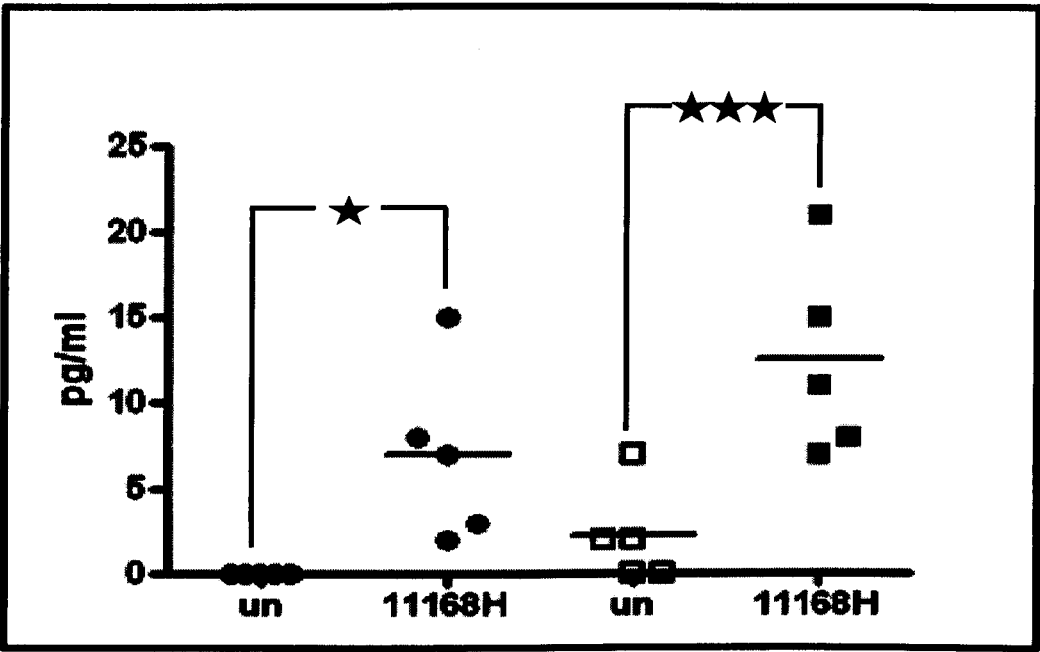


Figure 3.11: Human intestinal biopsies from the ileum (circles, N = 5) and the colon (squares, N = 5) were either mock infected with PBS (open symbols) or infected with *C. jejuni* 11168H (black symbols) wild-type strain for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of IL-17. * = $p < 0.05$, *** = $p < 0.001$. Adapted from Edwards *et al.* 2010.

Taken together, the presence of IFN- γ and IL-17 suggests that the *C. jejuni* 11168H wild-type strain initiates a Th17 response in *ex vivo* infected human intestinal biopsies, supporting *in vitro* data obtained with the ICH collaborators.

3.10 Investigating the role of *C. jejuni* surface structures involved in eliciting the secretion of hBD-2 and hBD-3 from *ex vivo* infected human intestinal biopsies.

In addition to the investigation of the host innate immune response induced by *ex vivo* infection of human intestinal biopsies with *C. jejuni* wild-type strains, a further study was undertaken to analyse the role of two *C. jejuni* surface structures in inducing the host innate immune response during *ex vivo* infection.

The identification of pathogenic bacteria by the host via recognition of bacterial surface structures is well established (Heine *et al.*, 2005). The bacterial surface structures recognised include flagellins, LPS/LOS, lipoproteins and peptidoglycan. In order to identify which *C. jejuni* MAMPs are recognised by the host and lead to the induction of an innate immune response in the EVOC model, the induction of hBD-2 and hBD-3 by a *C. jejuni* 11168H *flaA* mutant lacking the major flagellar filament protein FlaA and a *C. jejuni* 11168H *neuBI* mutant lacking the sialic acid residue on the LOS were analysed in *ex vivo* infected human intestinal biopsies.

3.10.1 Absence of the major flagellar filament protein FlaA does not abrogate the hBD-2 and hBD-3 response of *ex vivo* infected human intestinal biopsies to *C. jejuni* 11168H

Flagellin proteins have been demonstrated to be one of the bacterial MAMPs recognised by the host for several pathogenic microorganisms (Smith *et al.*, 2002). Toll-like receptor 5 (TLR5) has been demonstrated to be the host receptor responsible for recognition of flagellin from both Gram-positive as well as Gram-negative bacteria (Hayashi *et al.*, 2001). The amino acid residues responsible for flagellin-TLR5 binding have been mapped for both the human TLR5 receptor (Mizel *et al.*, 2003) as well as the flagellin protein (Smith *et al.*, 2003). However, sequence analysis demonstrated that *C. jejuni* flagellin lacks the amino acids recognised by TLR5 and subsequent experimental data has shown that *C. jejuni* flagellin is not recognised by TLR5 (Andersen-Nissen *et al.*, 2005, Watson *et al.*, 2005). Recombinant insertion of defined regions of *Salmonella* flagellin into the *C. jejuni* flagellin restored recognition of *C. jejuni* flagellin by TLR5 (de Zoete *et al.*, 2010). A *C. jejuni* *flaA* mutant has been shown to be non-motile, to be less able to invade IECs *in vitro* compared to the wild-type strain, but to still be able to secrete the Cia proteins from the stunted flagellum (Wassenaar *et al.*, 1991, Konkel *et al.*, 2004). Recent evidence has suggested that a *C. jejuni* 81-176 *flaA* mutant is unable to adhere to and form biofilms on *ex vivo* infected human intestinal tissue as assessed by microscopy (Haddock *et al.*, 2010). In *Salmonella*, the flagella have not only been demonstrated to be an important MAMP for the pro-inflammatory host response, but have also been indicated to be involved in dampening apoptotic processes in infected

intestinal epithelium (Vijay-Kumar *et al.*, 2006). As such the ability of a *C. jejuni* 11168H *flaA* mutant to elicit an hBD response from *ex vivo* infected human intestinal biopsies was analysed.

3.10.2 Absence of the major flagellar filament protein FlaA does not abrogate the hBD-2 response of *ex vivo* infected human intestinal biopsies to *C. jejuni* 11168H

Human intestinal biopsies from the ileum and the colon were either mock infected with PBS or infected with *C. jejuni* 11168H wild-type strain or the 11168H *flaA* mutant for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of hBD-2 (Figure 3.12)

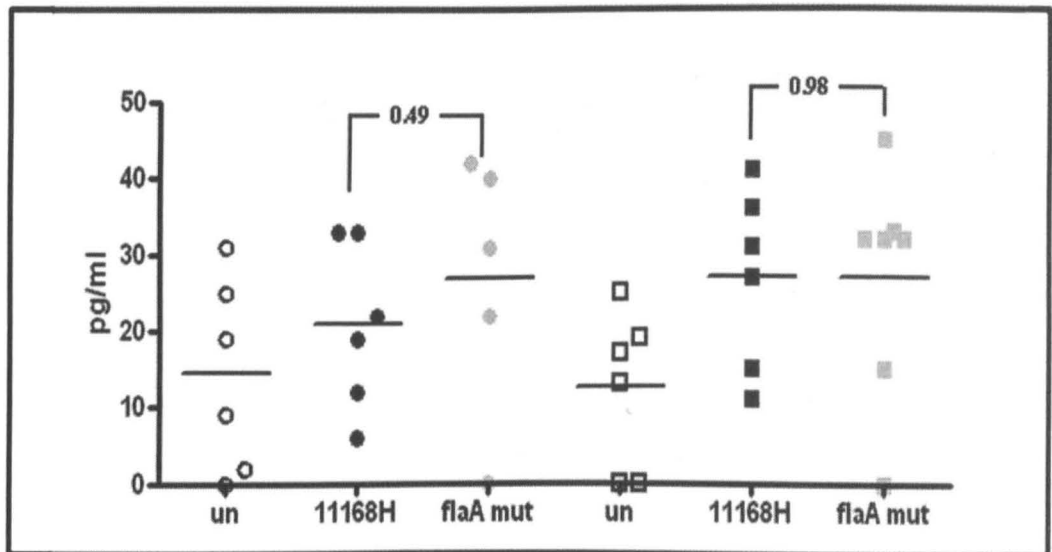


Figure 3.12: Human intestinal biopsies from the ileum (circles, N = 4 and 6) and the colon (squares, N = 6) were either mock infected with PBS (open symbols) or infected with *C. jejuni* 11168H wild-type strain (black symbols) or the 11168H *flaA* mutant (grey symbols) for 8 h *ex vivo*. The supernatants were probed for the presence of hBD-2.

No statistically significant difference between the amount of hBD-2 secreted in response to either *C. jejuni* 11168H wild-type strain or the 11168H *flaA* mutant was detected, indicating that the presence of the major flagellar filament protein FlaA is not required to trigger an hBD-2 response from the *ex vivo* infected human intestinal tissue (Figure 3.12).

3.10.3 Absence of the major flagellar filament protein FlaA does not
abrogate the hBD-3 response of *ex vivo* infected human intestinal
biopsies to *C. jejuni* 11168H

Human intestinal biopsies from the ileum and the colon were either mock infected with PBS or infected with *C. jejuni* 11168H wild-type strain or with the 11168H *flaA* mutant for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of hBD-3 (Figure 3.13)

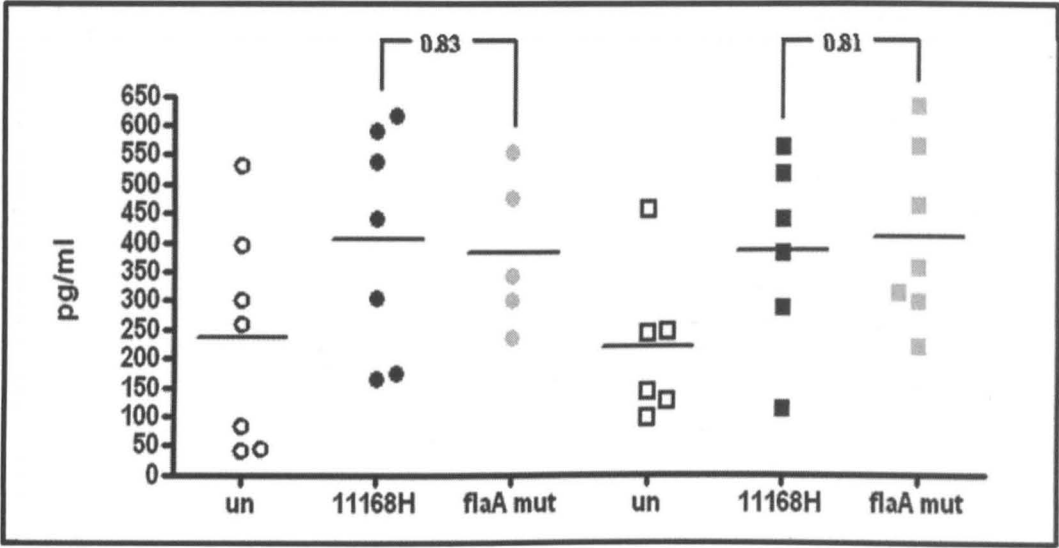


Figure 3.13: Human intestinal biopsies from the ileum (circles, N = 7 and 5) and the colon (squares, N = 6 and 7) were either mock infected with PBS (open symbols) or infected with *C. jejuni* 11168H wild-type strain (black symbols) or the 11168H *flaA* mutant (grey symbols) for 8 h *ex vivo*. The supernatants were probed for the presence of hBD-3

No statistically significant difference between the amount of hBD-3 secreted in response to either *C. jejuni* 11168H wild-type strain or the 11168H *flaA* mutant was detected, indicating that the presence of the major flagellar filament protein FlaA is not required to trigger an hBD-3 response from the *ex vivo* infected human intestinal tissue (Figure 3.13). Intriguingly, the reported reduced interactions of a *C. jejuni* 81-176 *flaA* mutant with human intestinal tissue (Haddock *et al.*, 2010) does not appear to have an effect on the induction of a defensin response.

3.10.4 Absence of the sialic acid residue on *C. jejuni* 11168H LOS does not abrogate the hBD-2 and hBD-3 response of *ex vivo* infected human intestinal biopsies

Bacterial LPS or LOS has been shown to be a potent mediator of host innate immunity. Furthermore, the LOS of certain *C. jejuni* strains has been shown to mimic ganglioside sugars found in humans. This has been implicated in the development of post-infectious sequelae, the most important being GBS (see section 1.1.6.2). An important feature of the LOS of certain *C. jejuni* strains is the presence of a sialic acid residue. The presence of this residue has recently been shown to mediate TLR4-dependent sensing of *C. jejuni* by DCs (Kuijf *et al.*, 2010).

To investigate whether the presence of the sialic acid residue in the *C. jejuni* 11168H wild-type strain LOS was important for induction of an hBD response from *ex vivo* infected human intestinal biopsies, EVOC experiments were performed using a *C. jejuni* 11168H *neuB1* mutant. NeuB1 has been shown to act as a sialic acid synthetase in sialation of LOS and the *neuB1* mutant has been shown to produce a truncated LOS lacking the sialic acid residue (Linton *et al.*, 2000b).

3.10.5 Absence of the sialic acid residue on *C. jejuni* 11168H LOS does not abrogate the hBD-2 response of *ex vivo* infected human intestinal biopsies

Human intestinal biopsies from the ileum and the colon were either mock infected with PBS or infected with *C. jejuni* 11168H wild-type strain or with the 11168H *neuB1* mutant for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of hBD-2 (Figure 3.14).

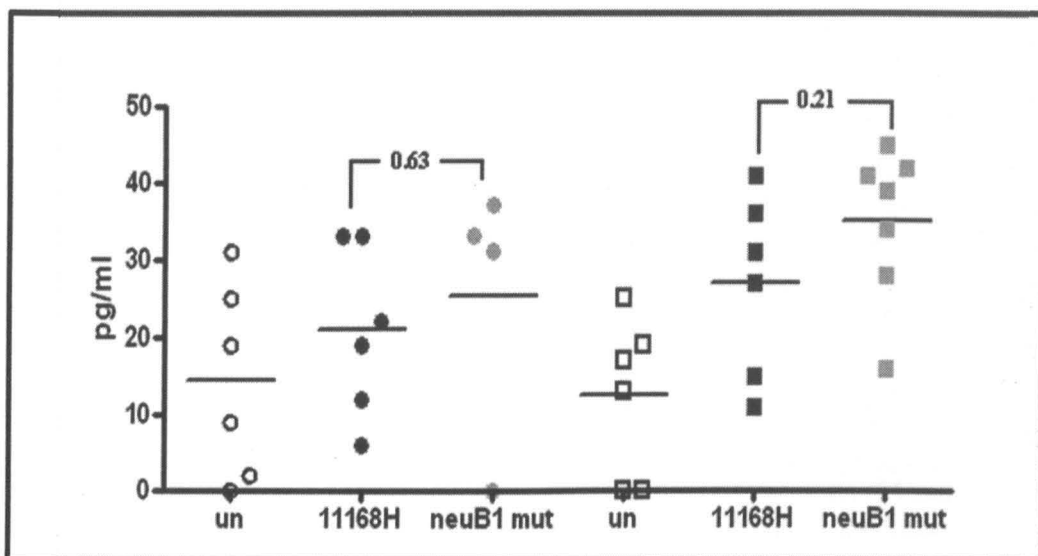


Figure 3.14: Human intestinal biopsies from the ileum (circles, N = 6 and 4) and the colon (squares, N = 6 and 7) were either mock infected with PBS (open symbols) or infected with *C. jejuni* 11168H wild-type strain (black symbols) or the 11168H *neuB1* mutant (grey symbols) for 8 h *ex vivo*. The supernatants were probed for the presence of hBD-2.

No statistically significant difference between the amount of hBD-2 secreted in response to either *C. jejuni* 11168H wild-type strain or the 11168H *neuB1* mutant was detected (Figure 3.14). However, a trend was observed in the colonic samples, where approximately 1.25 times more hBD-2 was detected in the samples infected with the 11168H *neuB1* mutant. This was not observed for the *flaA* mutant (see Figure 3.12).

3.10.6 Absence of the sialic acid residue on *C. jejuni* 11168H LOS does not abrogate the hBD-3 response of *ex vivo* infected human intestinal biopsies

Human intestinal biopsies from the ileum and the colon were either mock infected with PBS or infected with *C. jejuni* 11168H wild-type strain or with the 11168H *neuB1* mutant for 8 h *ex vivo*. The supernatants were probed by ELISA for the presence of hBD-3 (Figure 3.15).

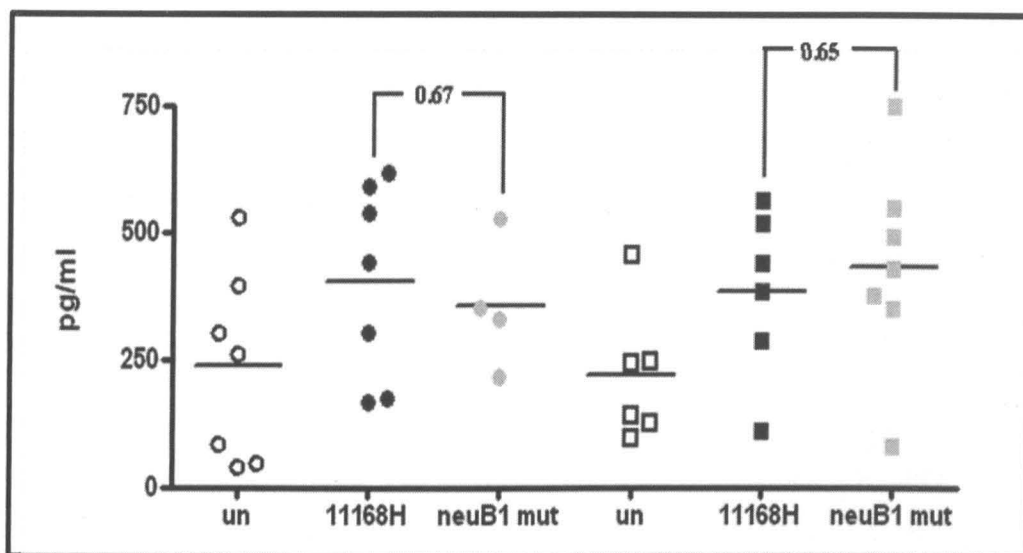


Figure 3.15: Human intestinal biopsies from the ileum (circles, N = 7 and 4) and the colon (squares, N = 6 and 7) were either mock infected with PBS (open symbols) or infected with *C. jejuni* 11168H wild-type strain (black symbols) or the 11168H *neuB1* mutant (grey symbols) for 8 h *ex vivo*. The supernatants were probed for the presence of hBD-3.

No statistically significant difference between the amount of hBD-3 secreted in response to either *C. jejuni* 11168H wild-type strain or the 11168H *neuB1* mutant was detected (Figure 3.15). In contrast to the data obtained for hBD-2, no increase was observed in the supernatants of colonic tissue infected *ex vivo* with the 11168H *neuB1* mutant. This suggests that the presence of the sialic acid residue on the *C. jejuni* 11168H LOS is not essential for eliciting an hBD-2 and hBD-3 response in *ex vivo* infected human intestinal tissue. Whether the trend observed with hBD-2 but not with hBD-3 in the colonic supernatants reflects a biologically significant result is unclear due to the relatively low numbers of replicates.

3.11 Summary and discussion

The aim of this study was to assess the feasibility of an EVOC model to investigate *C. jejuni* host-pathogen interactions using primary human intestinal biopsies from the colon and terminal ileum. Two studies have demonstrated physical interaction of *C. jejuni* with human intestinal tissue *ex vivo* (Grant *et al.*, 2006, Haddock *et al.*, 2010) and one study has demonstrated secretion of the pro-inflammatory chemokine IL-8 by intestinal tissue in response to *ex vivo* infection with *C. jejuni* (MacCallum *et al.*, 2006). One of the advantages of the EVOC model using whole primary tissue is

the fact that all cell types found *in vivo* in the intestine are present in the tissue and barring major damage during removal of the tissue are in the same arrangement. Disadvantages include the difficulty of obtaining intestinal tissue, as the research institute must be linked with a gastroenterology ward at a hospital. Even then, the consent of the patients/parents in the case of paediatric tissue must be obtained. Furthermore, the lifespan of such tissue *ex vivo* is limited.

These experiments also presented multiple challenges in terms of the number of replicates possible and the technical difficulties with splitting samples for comparative studies. The appropriate statistical analyses for such data was carefully considered. Consultation of a medical statistician (Timothy Collier, LSHTM) resulted in the suggestion that an alternative to analysis of the data by Student's t-test should be explored. It was suggested that matched pairs of uninfected and infected biopsies from the same donor are tested in a paired fashion may be a more appropriate method of analysis, as this would represent a "like with like" analysis. However, the sizes of the biopsies varied considerably, so that comparison of two pieces of tissue from the same patient would not necessarily represent a "like with like" comparison due to the different numbers of cytokine and defensin secreting cells. Unfortunately, no size or weight measurements of the tissues were recorded when the infections were performed, rendering a retrospective normalisation of tissue pairs inappropriate. In these circumstances, the independent grouping of uninfected and infected tissue is the most appropriate approach to the statistical analysis of this data. As such, the suggestion that a non-parametric test such as the Wilcoxon U-test may be advantageous over the student's t-test due to the distribution of some of the data was made. Indeed the Wilcoxon U-test has been used by collaborators at ICH following discussions with their in-house medical statisticians (Edwards *et al.*, 2010). Therefore, all the datasets were retested using the Wilcoxon U-test (data not shown). This statistical reanalysis of the datasets did not alter any of the statistical outcomes indicated in the results presented in this chapter, suggesting that the student's t-test was an appropriate statistical analysis to use under these circumstances.

In order to achieve more of a "like with like" comparison for future studies, one of two modifications to the experimental setup could be applied. Firstly any given piece of tissue could be cut into two equal halves, leaving one uninfected and infecting the other, thereby creating matched pairs. Secondly, if the tissue pieces are too small to

be cut (as was often the case with paediatric biopsies), the weight of each tissue piece should be determined prior to commencing the EVOC experiments. This would allow the protein levels detected by ELISA in the supernatants to be normalised against the weight of the tissue and provide a much better data set for analysis by the recommended matched pair approach.

Initial experiments were performed in order to reproduce the published data demonstrating the secretion of IL-8 from human intestinal biopsies after *ex vivo* infection with *C. jejuni* strains NCTC11168 and L115 (MacCallum *et al.*, 2006). However, in contrast to the published data, no difference was detected between the IL-8 present in the supernatants of uninfected and infected tissue (Figure 3.2 and 3.3). This could be attributed to several factors. Firstly the origin and preparation of the tissue for *ex vivo* culturing differed in several aspects. In the previous study, two large tissue samples from two patients were obtained and cut into several small pieces for *ex vivo* infection. This lowers the sample number extensively and may skew any results obtained due to the lack of person to person variation. In this study, each data point represents one individual patient and as such a much larger sample number is covered. Assuming that some of the patients undergoing endoscopy had increased IL-8 levels present in their intestine, the chances of including more of these in a larger sample number is increased. This would in turn lead to the basal levels of uninfected tissue already being high and therefore masking any effect of exposure of the tissue to *C. jejuni*, leading to potential false negative results. Secondly, in the previous study the tissue was transported from the endoscopy suite to the laboratory in medium containing antibiotics in order to remove the intrinsic microflora. As no overgrowth was observed during the initial experiments performed in this study and as the original report of an EVOC model did not include antibiotics in the transport medium (Hicks *et al.*, 1996), the tissue was transported to the laboratory in medium without antibiotics. Removing the natural microflora present on the tissue is also counterproductive to one of the advantages of the EVOC model, namely to stay as close as possible to the *in vivo* situation. There is evidence that the presence of commensal intestinal bacteria is involved in attenuating inflammation (Kelly *et al.*, 2004). The presence or absence of the microflora during the *ex vivo* co-culturing of the tissue with *C. jejuni* might also influence the production of IL-8, providing one explanation for the difference between the data presented here and the

previously published work. Thirdly, another difference between the previous study and the work presented here is the source of the human intestinal tissue. Whilst tissue from adult patients was used in the previous work, the work presented here utilised paediatric tissue. This is another source of variation between the two studies, and may have led to the observed differences between this study and previously published work in terms of basal levels of IL-8 secretion as well as inducibility of IL-8 *per se* from *ex vivo* infected human tissue.

Previous work demonstrated the production of hBD-2 and hBD-3 in response to *C. jejuni* infection of IEC monolayers *in vitro* (Zilbauer *et al.*, 2005). As opposed to the data obtained for IL-8, analysis of the EVOC supernatants for the presence of the two defensin peptides revealed increased amounts of both peptides in the supernatants of tissue infected with two different *C. jejuni* wild-types strains, 11168H and 81-176. Significant differences between the overall levels of hBD-2 and hBD-3 detected in the infected supernatants (around 25 pg/ml for hBD-2 versus around 400 pg/ml for hBD-3) may be due to the affinity of hBD-2 for the polypropylene plastic of the tubes used for storage of the supernatants and therefore reflect an artefact of the experiment rather than the biological situation (Mark Lucas, PhD thesis, University of London, 2010). Concentration of the supernatants using Amicon centrifugal filter units may allow for the detection of hBD-2 if the concentration in the neat sample is below the detection limit of the ELISA. This *ex vivo* data highlights the role for the defensins in the host innate immune response to *C. jejuni* infection. Furthermore, it is the first time that secretion of hBD-2 and hBD-3 has been demonstrated in response to *ex vivo* infection of human intestinal biopsies with *C. jejuni*. Further investigation into this finding, for example by analysis of the supernatants for other defensin peptides, would be merited.

Similar to the data obtained for hBD-2 and hBD-3, higher levels of IL-1 β and IL-6 were detected in the supernatants of infected biopsies compared to the uninfected controls. This was the first time that these cytokines have been detected from human intestinal tissue infected *ex vivo* with *C. jejuni* and supports data from other studies suggesting that these cytokines are involved in the host innate immune response to *C. jejuni* (Friis *et al.*, 2009, Siegesmund *et al.*, 2004).

Data from collaborators suggested that *C. jejuni* 11168H wild-type strain infection of DCs leads to the secretion of cytokines that, when co-incubated with naïve T-cells, lead to the development of a mixed Th1 / Th17 adaptive immune response (Edwards

et al., 2010). To assess whether the activation of a Th17 response could be detected in *ex vivo* infected tissue, the supernatants were screened for the presence of IL-12 and IL-23. Statistically significant higher levels of both IL-12 and IL-23, as well as IL-17 and IFN- γ , were detected in infected biopsies compared to the uninfected controls, suggesting that *C. jejuni* infection does not only lead to the upregulation of defensins in *ex vivo* infected human intestinal tissue, but also triggers a Th1 / Th17 adaptive immune response.

It is still not fully understood which part of the *C. jejuni* cell is recognised by the immune system. Some evidence suggests that the bacterial flagellum, an important structure involved in the recognition of other pathogenic bacteria such as *Salmonella* species is not involved in the recognition of *C. jejuni*. This was confirmed using a *C. jejuni* 11168H *flaA* mutant lacking the major flagellar filament protein FlaA, which elicited a similar level of defensin response as the *C. jejuni* 11168H wild-type strain. As the 11168H *flaA* mutant still possesses stunted flagella comprised of the FlaB flagellin protein and is still able to secrete proteins, it would be interesting to analyse a “more complete” aflagellate mutant in the EVOC model system. One suitable mutant would be the 11168H *rpoN* (σ^{54}) mutant, which has been demonstrated to be completely aflagellate and secretion-negative (Fernando *et al.*, 2007). Intriguingly, a recent report demonstrated that a *C. jejuni* 81-176 *flaA* mutant was less able to adhere to and form microcolonies and biofilms on primary human intestinal tissue (Haddock *et al.*, 2010). Unfortunately, that study did not analyse the innate immune response elicited by the wild-type and mutant bacteria, whilst this study did not analyse the effect of the 11168H *flaA* mutation on the “direct” bacteria-tissue interactions. It would therefore be interesting to analyse the interaction of the 11168H *flaA* mutant with the intestinal tissue by microscopy to determine whether the induced hBD-2 and hBD-3 response remained the same as that induced by the wild-type strain despite reduced interactions. As the study presented here only analyses the levels of hBD-2 and hBD-3 at one time point after infection, an analysis of any potential time-dependence of the response, in particular to the 11168H *flaA* mutant, may shed further light on this question. Furthermore, construction and use of a 81-176 *flaA* mutant may highlight potential differences between the two wild-type strains.

Another bacterial surface structure that is recognised by the host is LOS. A 11168H *neuB1* mutant lacking the sialic acid residue of the wild-type LOS was shown to

elicit a similar level of defensin response from the tissue as the wild-type strain, suggesting that sialic acid residue of the LOS was not involved in modulating the response. As some recent evidence has suggested that the LOS may however, still be involved in pathogen recognition (Heikema *et al.*, 2010), it would be interesting to analyse the effect of more truncated LOS structures, or even of purified LOS from the wild type and LOS mutants on defensin expression. *C. jejuni* 11168H *waaC* and *waaF* mutants were constructed and preliminarily analysed, but unfortunately not studied in the EVOC model. WaaC and WaaF are two glycosyl transferases involved in biosynthesis of the *C. jejuni* LOS (Kanipes *et al.*, 2008).

Taken together, the data presented here demonstrates that *in vitro* infected human intestinal biopsies respond to *C. jejuni* challenge and could be used to further study the interactions of this important human pathogen with the host.

The model does however have certain limitations that cannot be overlooked. Firstly, human intestinal tissue is difficult to obtain, as close collaboration with a gastroenterologist and consent of the endoscopy patients are required, so experiments must be planned meticulously in order not to waste the available tissue. This also limits the number of replicates that can be performed. This limits the wider use of the model, as only few laboratories will have access to such tissue. Together with the possibility for person to person variation, this may somewhat limit the feasibility of the model. One of the advantages of the model, namely that the tissue is very close to the *in vivo* status and contains all cell types present in the gut, does also lead to another drawback, as any signal detected in the supernatant cannot be attributed to a specific subset of cells. Fractionation of the tissue followed by subsequent infection of the individual sub-fractions may partly correct for this, however, this will counteract one of the advantages of the model. Further studies using defined *C. jejuni* mutants may shed more light on the interaction between *C. jejuni* and the host tissue and help elucidate what bacterial structures are involved.

The data presented here demonstrates that the EVOC model is a useful model for studying the host innate immune response to *C. jejuni*. There are, however, several limitations to the model that need to be acknowledged and kept in mind when using the model

Chapter 4

Development of the Vertical Diffusion
Chamber model for co-culturing *C. jejuni*
with intestinal epithelial cells with
microaerobic conditions at the apical surface

4.1 Introduction

One major drawback of using *in vitro* cultured IECs for co-culturing experiments with *C. jejuni* is the aerobic environment commonly used during co-culturing. As well as not being a good model of the micro- to anaerobic environment present in the intestinal lumen, the aerobic co-culturing conditions might have detrimental effects on the microaerophilic *C. jejuni*. As such, the interactions of *C. jejuni* with *in vitro* cultured IECs observed during the co-culturing experiments may be a poor representation of what occurs *in vivo*. To counteract this, a Vertical Diffusion Chamber (VDC) was adapted to serve as a co-culturing model. A VDC is composed of two plastic half-chambers. The two chambers are clamped together, leaving space for a permeable plastic support (Snapwell™ filter) at the interface (Figure 4.1).

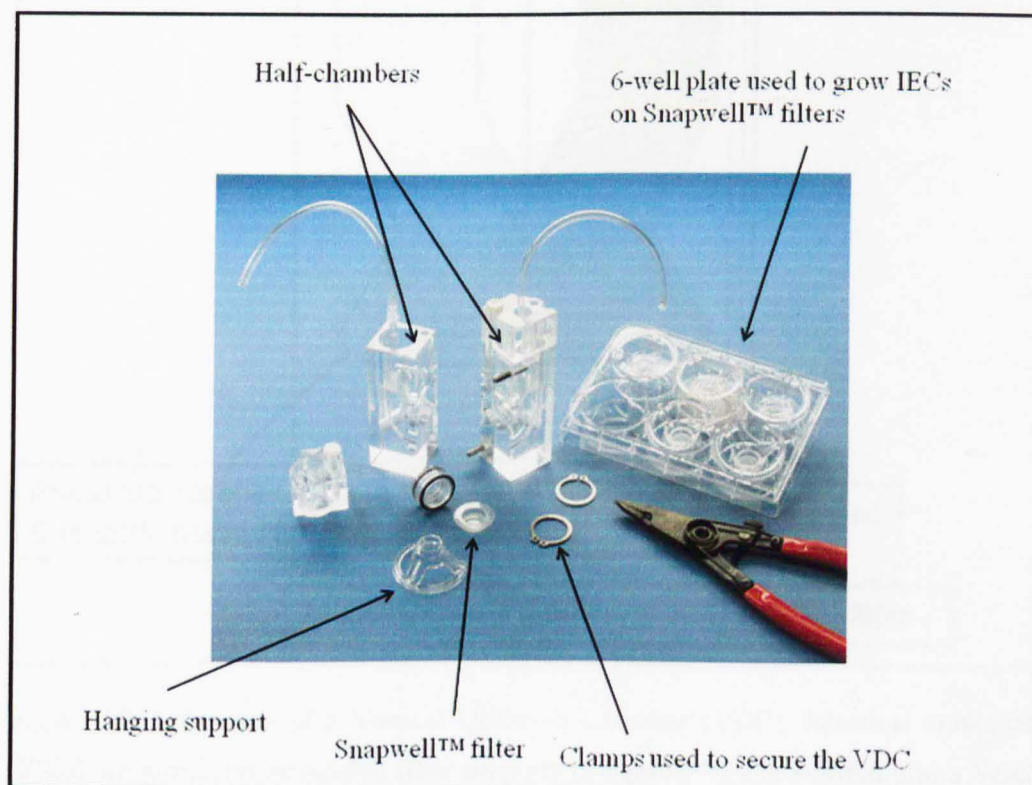


Figure 4.1: Image of a Vertical Diffusion Chamber (VDC) with the two half-chambers separated. The 6-well plate and the hanging support structure used for growing the IECs on Snapwell™ filters as well as for fluorescently labelled dextran diffusion assays are visible. Obtained from www.warneronline.com

Once a Snapwell™ filter is inserted, the two compartments are physically separated. The principle behind using the VDC to co-culture *C. jejuni* with IECs is the assumption that the IECs grown on Snapwell™ filters will act as a barrier and create separate apical and basolateral compartments. The conditions in the two compartments can then be individually adjusted in terms of both the medium and the gas composition. (Figure 4.2)

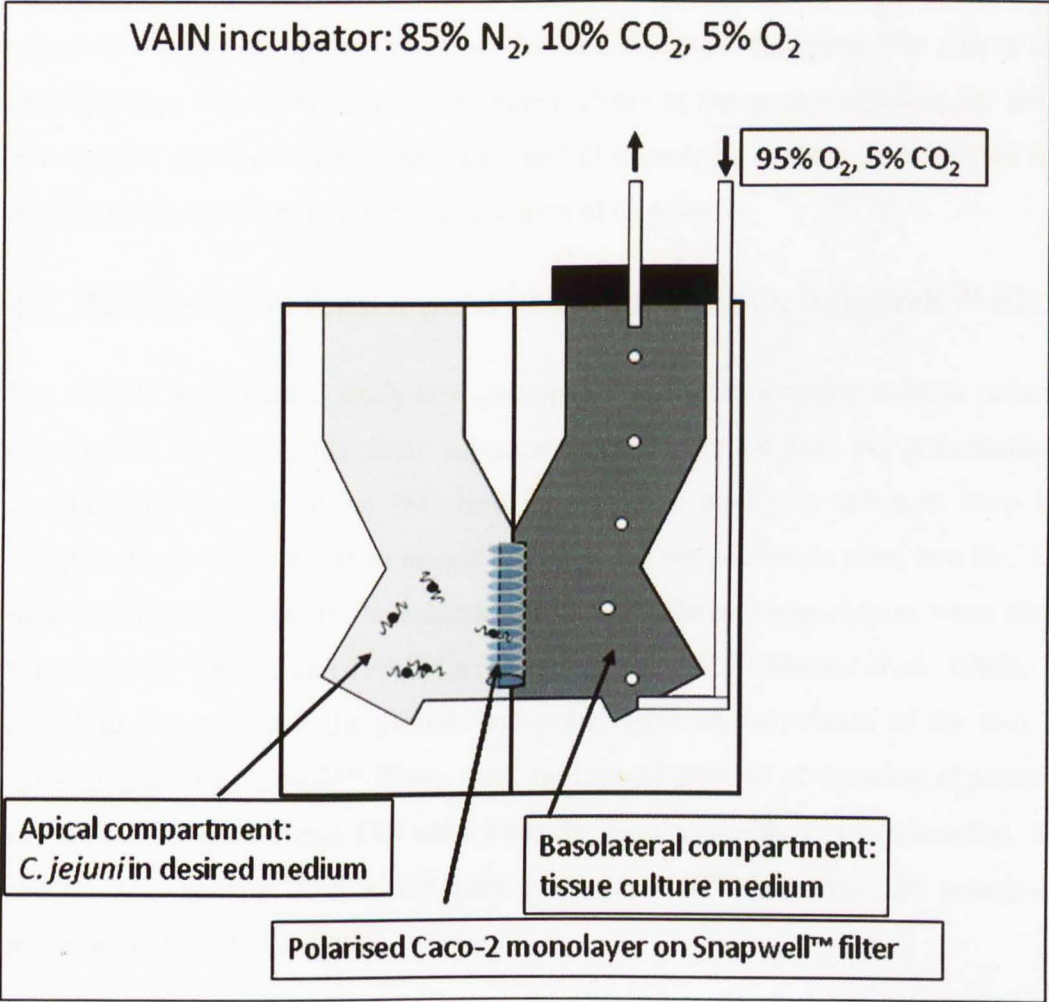


Figure 4.2: Schematic of a Vertical Diffusion Chamber (VDC). Intestinal epithelial cells (IECs) are grown on permeable filter supports (Snapwell™) and inserted into a VDC, thus creating an apical and a basolateral compartment. These compartments can then be individually adjusted with respect to medium and gas composition to allow the co-culturing of the IECs with *C. jejuni* with microaerobic conditions at the apical surface of the IECs and aerobic conditions at the basolateral surface of the IECs.

For co-culturing of *C. jejuni* with IECs, the basolateral compartment was filled with tissue culture medium and perfused with a gas mixture containing 95% O₂ and 5% CO₂ to allow oxygen supply to the IECs. As the IECs obtain nutrients from the basolateral side, the hypothesis was that the apical compartment could be filled with media that alone would not support the growth of IECs, such as bacterial growth media. More importantly, by placing the VDC into a microaerobic incubator and leaving the apical compartment open, the gas composition on the apical side can be adjusted to allow for optimal microaerobic conditions for *C. jejuni*. The aim of these modifications was to create an environment closer to the *in vivo* situation for the co-culturing of the bacteria with the IECs and ultimately to analyse the effect of these conditions on the pathogen-host interactions of *C. jejuni*.

4.2 IECs grow to form a polarised monolayer on Snapwell™ filters

Not all IEC lines used to study host-pathogen interactions are able to form polarised monolayers on permeable filter supports (see section 1.4.2.2). As polarisation is essential for the use of an IEC line in the VDC model in order to keep both compartments separate and to mimic the intestinal epithelium *in vivo*, two IEC lines that have been previously demonstrated to form polarised monolayers were chosen for this study; Caco-2 and T84 IECs (Madara *et al.*, 1987, Chantret *et al.*, 1988).

Initial studies to assess the growth and polarisation characteristics of the two IEC lines grown on Snapwell™ filters were performed prior to performing experiments in the VDCs. Caco-2 and T84 cells from the lines available in the laboratory were seeded at a density of 5×10^5 cells per Snapwell™ filter and the polarisation monitored for up to 21 days.

4.2.1 Analysis of the permeability of IEC monolayers after 21 days of growth by fluorescent dextran diffusion indicates the formation of an impermeable monolayer.

The permeability of an IEC monolayer can be assessed by monitoring the diffusion of a fluorescently labelled dextran tracer across the monolayer. This diffusion was assessed for both Caco-2 and T84 cells that had been grown for 21 days on permeable Snapwell™ filters. For the dextran diffusion assay, the Snapwell™ filters

were kept on the hanging support structures that had been used for the growing of the IECs as shown in Figure 4.3. The principle behind the assay is that a fully polarised and impermeable IEC monolayer would retain all the tracer on the side the tracer was added to (A), whilst in the case of an incompletely polarised and therefore semi-permeable IEC monolayer, some of the fluorescently labelled tracer would pass across the monolayer (B)

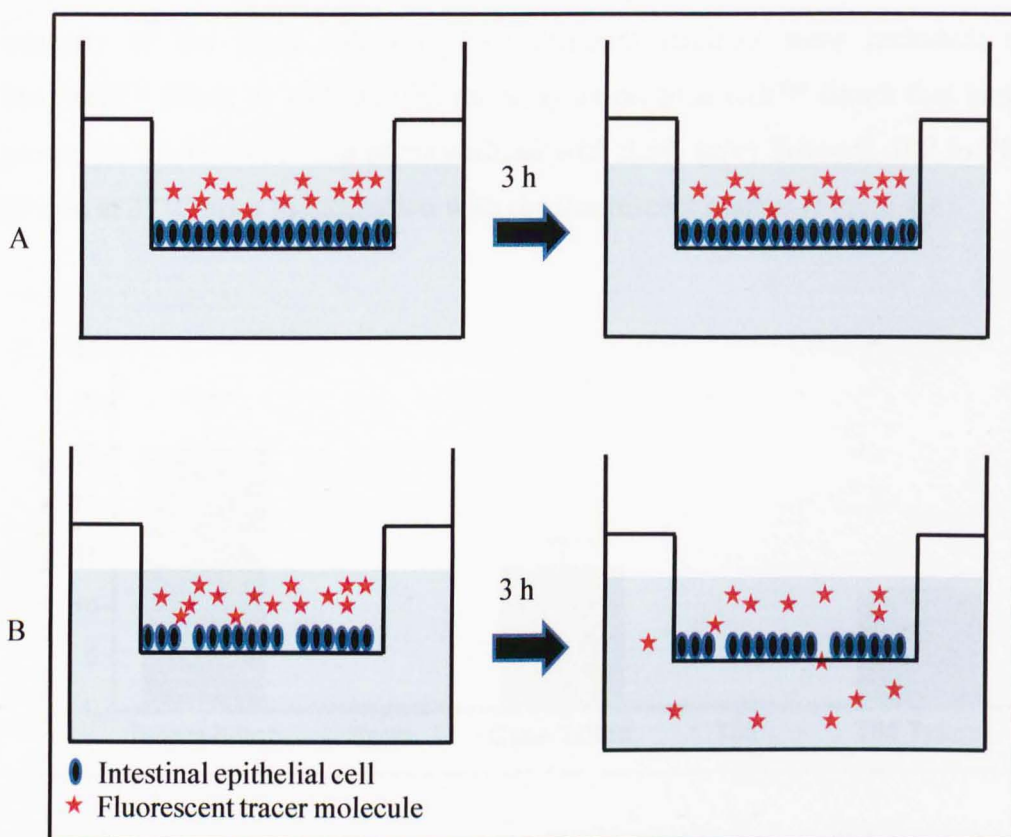


Figure 4.3: Diagram representing the fluorescent dextran diffusion assay. Fluorescently labelled dextran (asterisks) was added to the apical side of an IEC monolayer and incubated for 3 h. After the incubation, the amount of fluorescently labelled dextran that had crossed the monolayer to the basolateral side of the IECs was determined. If the IEC monolayer was fully confluent and polarised with tight cell-cell junctions, no fluorescently labelled dextran was expected to diffuse into the basolateral compartment (A). In the case of a sub-confluent, non-polarised monolayer, some fluorescently labelled dextran was expected to be detected in the basolateral compartment (B).

IECs were grown on Snapwell™ filters for 21 days, washed three times with sterile PBS and 500 μ l of 100 μ M FITC-labelled dextran with an average molecular weight of 4 kilodaltons (kDa) in Ringer's solution was added to the apical side of the monolayer and incubated for 3 hours at room temperature, with the basolateral side of the monolayer immersed in Ringer's solution. The amount of fluorescently labelled dextran on the basolateral side of the monolayer was determined post-incubation by removal of the supernatant and measurement of the fluorescence intensity at 488 nm as described in section 2.10.7. The percentage of fluorescently labelled dextran that had crossed the IEC monolayer was calculated by dividing the fluorescence intensity recorded in the basolateral compartment by the fluorescence

intensity of the input solution. Two different controls were included; empty Snapwell™ filters as well as IEC monolayers on Snapwell™ filters that had been grown for 21 days but been permeabilised with 0.5% (v/v) Triton X-100 in PBS for 20 min at 37°C prior to incubation with the fluorescent marker (Figure 4.4).

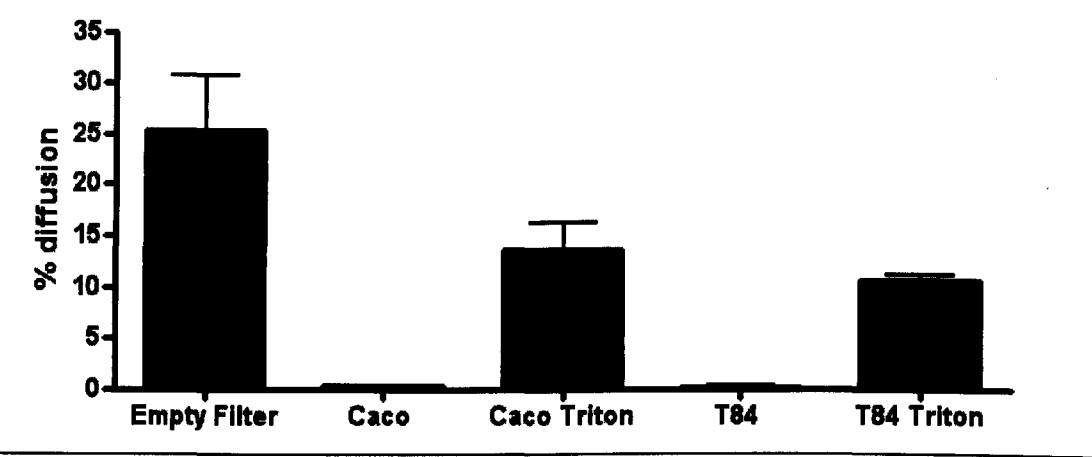


Figure 4.4: Analysis of the diffusion of a fluorescent marker across cellular monolayers on Snapwell™ filters. The intestinal epithelial cells (IECs) were grown on Snapwell™ filters for 21 days and a solution of fluorescently labelled dextran added to the apical side of the IECs. After 3 h, the % of diffusion of the fluorescently labelled dextran across the monolayer was determined by measuring the relative fluorescence of the basolateral solution at 488 nm and dividing the value by the relative fluorescence of the input solution. An empty Snapwell™ filter and IECs grown for 21 days on Snapwell™ filters, then permeabilised with 0.5% (v/v) Triton X-100 for 20 min at 37°C were used as controls.

The diffusion analysis indicated that a polarised monolayer of both Caco-2 and T84 IECs formed a barrier that was non-permeable to the fluorescent tracer, indicating that the IECs had formed an impermeable monolayer. In the two control samples, diffusion of the fluorescent tracer across the filter was observed, with the highest diffusion occurring across the empty Snapwell™ filters. In addition, the assay confirmed that exposure of the IEC monolayer to a 0.5% (v/v) solution of Triton X-100 solution in PBS for 20 min at 37°C is sufficient to permeabilise the IECs.

4.2.2 Analysis of the Polarisation Status of IECs grown on Snapwell™ filters for up to 21 days by Laser Scanning Confocal Microscopy indicates the formation of a confluent, polarised monolayer.

Impermeability of the Caco-2 and T84 IEC monolayers to fluorescently labelled dextran suggested the formation of a tight, polarised monolayer on Snapwell™ filters over a period of 21 days. Analysis of the distribution of brush border actin and of the tight junctional protein occludin was performed by Laser Scanning Confocal Microscopy (LSCM). IECs were seeded on three Snapwell™ filters and one filter was removed and fixed with paraformaldehyde (PFA) after 7, 14 and 21 days respectively. The filters were stained for actin and occludin as described in section 2.10.6 and examined by LSCM. All figures represent *in silico* derived projections of Z-stacks, which was required as the tight junctions containing the occludin protein are located at different heights throughout the sample.

4.2.2.1 Caco-2 cells

After 7 days of growth, the Caco-2 cells on the filters had completely covered the filter. However, the cells only stained weakly for actin and occludin (Figure 4.5). Most of the actin was confined to cell-cell junctions, with no apical actin present. Occludin was observed at some of the cell-cell junctions. This suggested that 7 days of incubation was sufficient for the cells to spread over the filter, but not to form a tight, polarised monolayer. At 14 days post-seeding, a stronger actin stain was observed, with approximately 40% of the cells staining for actin on the apical surface, indicating the development of a brush border (Figure 4.6). Occludin staining was observed along the cell-cell junctions in approximately 30% of the cells. At 21 days post-seeding, strong apical actin staining was observed in approximately 80% of the cells, while about 90% of the cell-cell junctions stained positive for occludin (Figure 4.7).

Taken together, impermeability to fluorescent dextran and the LSCM analysis demonstrated that Caco-2 cells grew to form a impermeable, polarised monolayer on Snapwell™ inserts within 21 days of seeding.

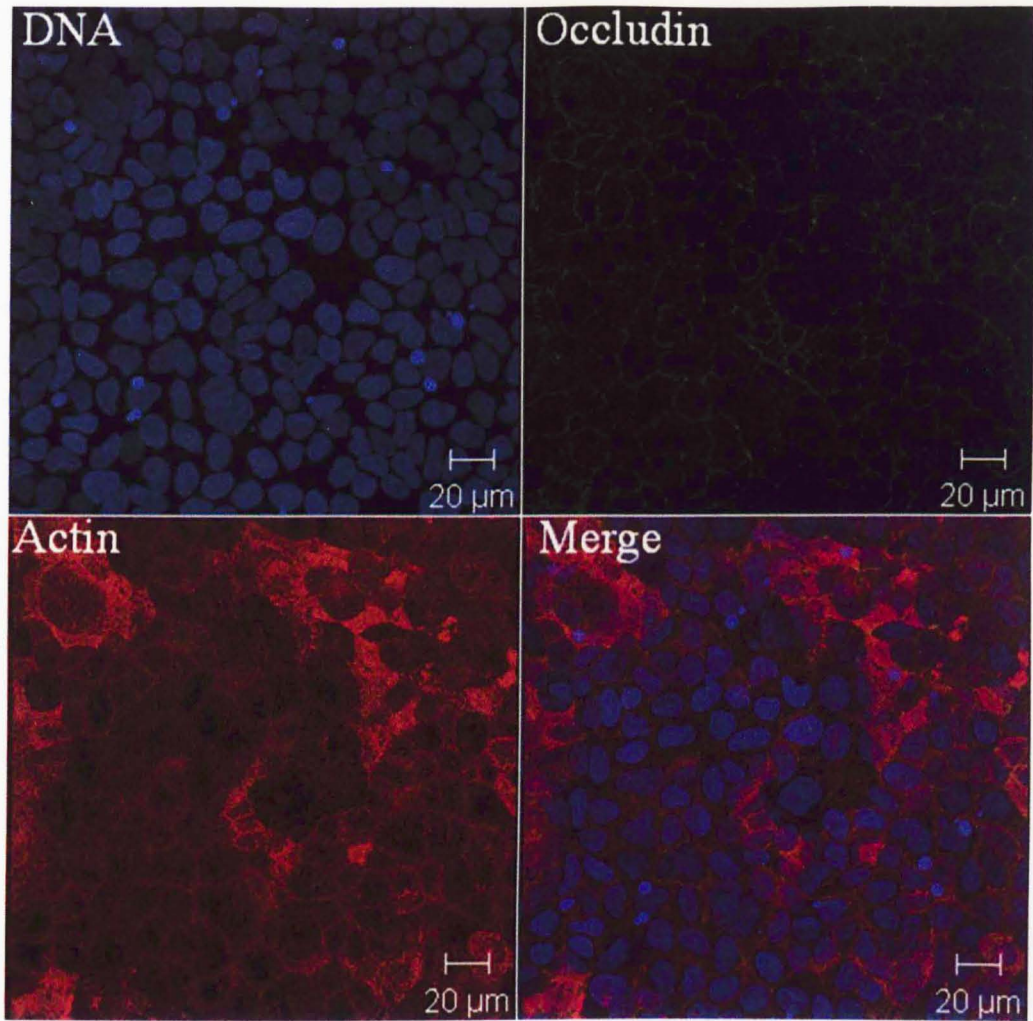


Figure 4.5: 5×10^5 Caco-2 intestinal epithelial cells (IECs) were seeded onto Snapwell™ filters. After 7 days of growth, a filter was removed and fixed in PFA. The cells were processed for immunostaining and stained for occludin (green), actin (red) and the nuclei counterstained with DAPI (blue). The images represent projections of a stack of Z-axis slices viewed from above.

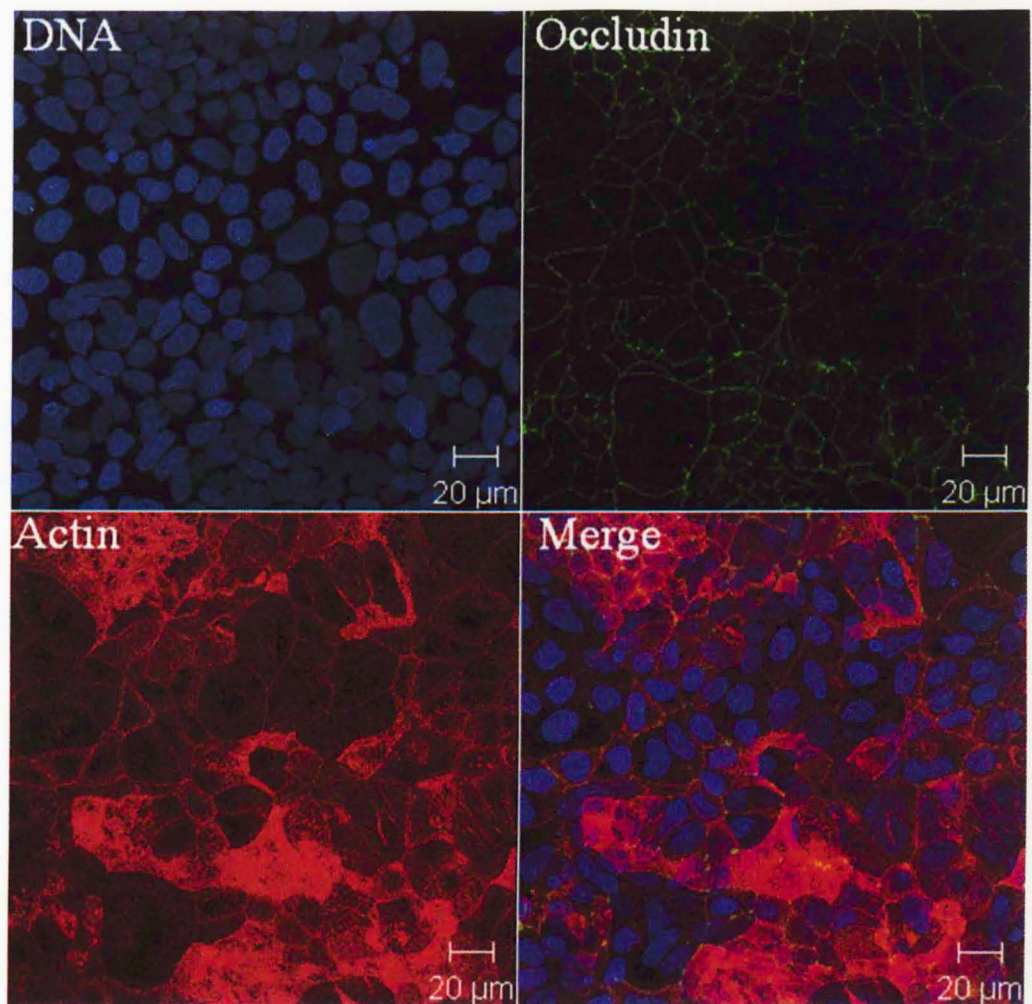


Figure 4.6: 5×10^5 Caco-2 intestinal epithelial cells (IECs) were seeded onto Snapwell™ filters. After 14 days of growth, a filter was removed and fixed in PFA. The cells were processed for immunostaining and stained for occludin (green), actin (red) and the nuclei counterstained with DAPI (blue). The images represent projections of a stack of Z-axis slices viewed from above.

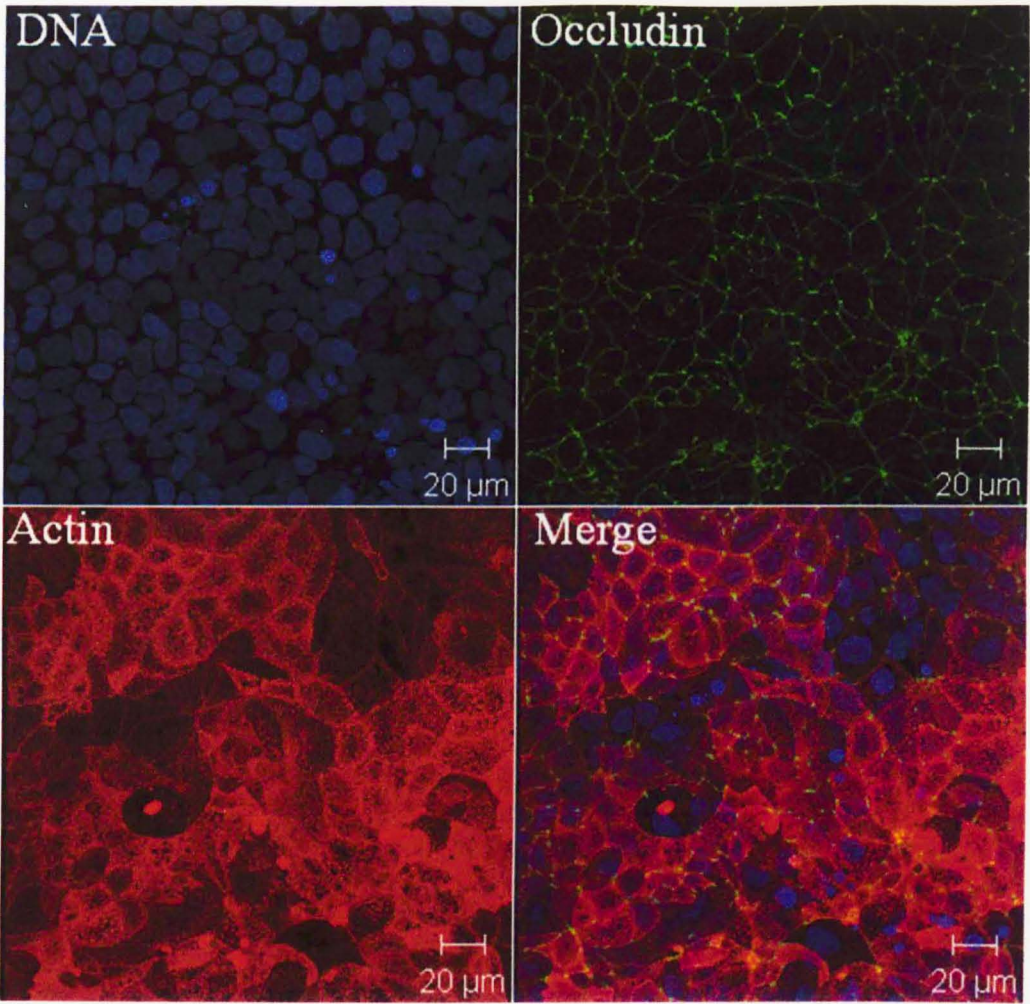


Figure 4.7: 5×10^5 Caco-2 intestinal epithelial cells (IECs) were seeded onto Snapwell™ filters. After 21 days of growth, a filter was removed and fixed in PFA. The cells were processed for immunostaining and stained for occludin (green), actin (red) and the nuclei counterstained with DAPI (blue). The images represent projections of a stack of Z-axis slices viewed from above.

4.2.2.2 T84 Cells

T84 cells were seeded at 5×10^5 cells per Snapwell™ and one Snapwell™ removed after 7, 14 and 21 days of growth respectively as described in section 4.1.2.1. Staining for actin and occludin indicated the presence of a weak occludin meshwork along the cell-cell junctions as early as 7 days post-seeding (Figure 4.8). The development of a complete occludin meshwork was demonstrated at 14 days post-seeding (Figure 4.9). Furthermore, a strong apical actin stain was observed at 14 days post-seeding, suggesting the development of a brush border. Incubation of the IECs for another 7 days did not dramatically change the appearance of the T84 IEC monolayer and was therefore deemed unnecessary (Figure 4.10). As a result, all further experiments with T84 cells were performed after 14 days of growth.

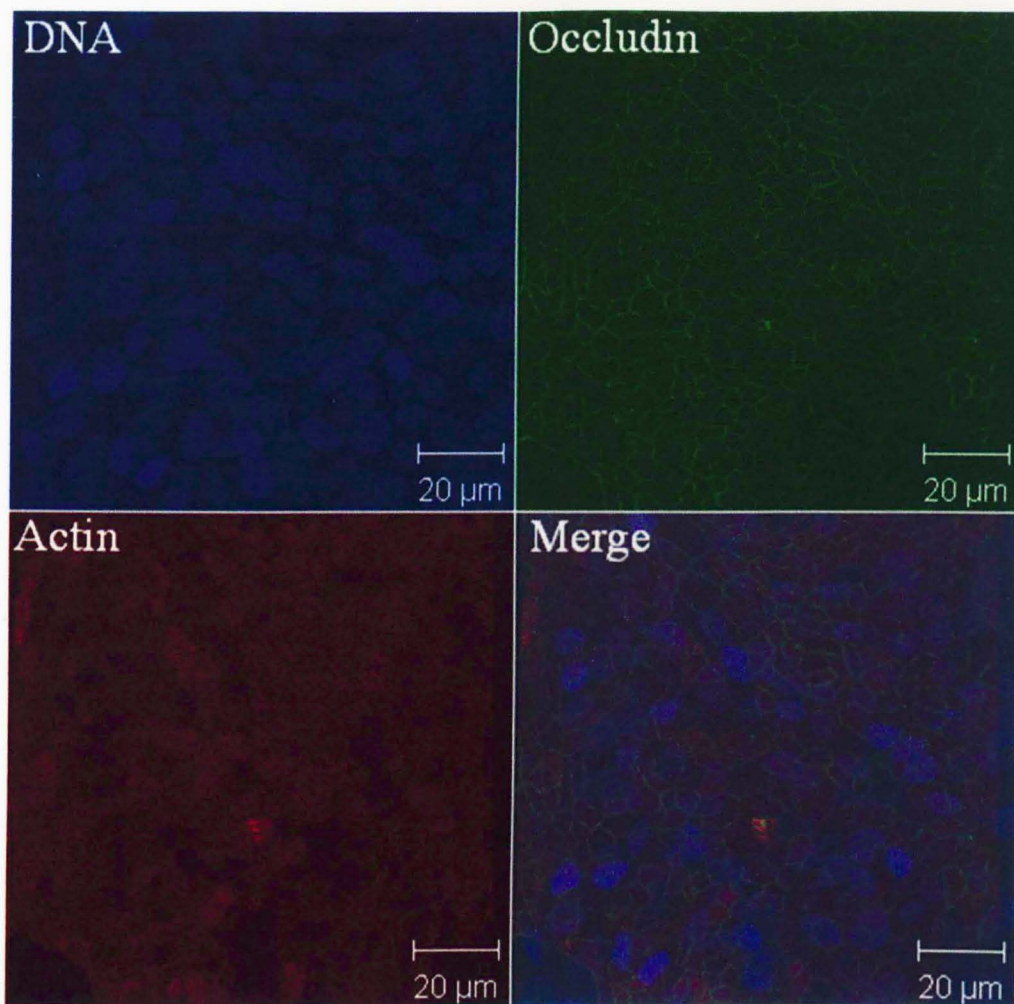


Figure 4.8: 5×10^5 T84 intestinal epithelial cells (IECs) were seeded onto Snapwell™ filters. After 7 days of growth, a filter was removed and fixed in PFA. The cells were processed for immunostaining and stained for occludin (green), actin (red) and the nuclei counterstained with DAPI (blue). The images represent projections of a stack of Z-axis slices viewed from above.

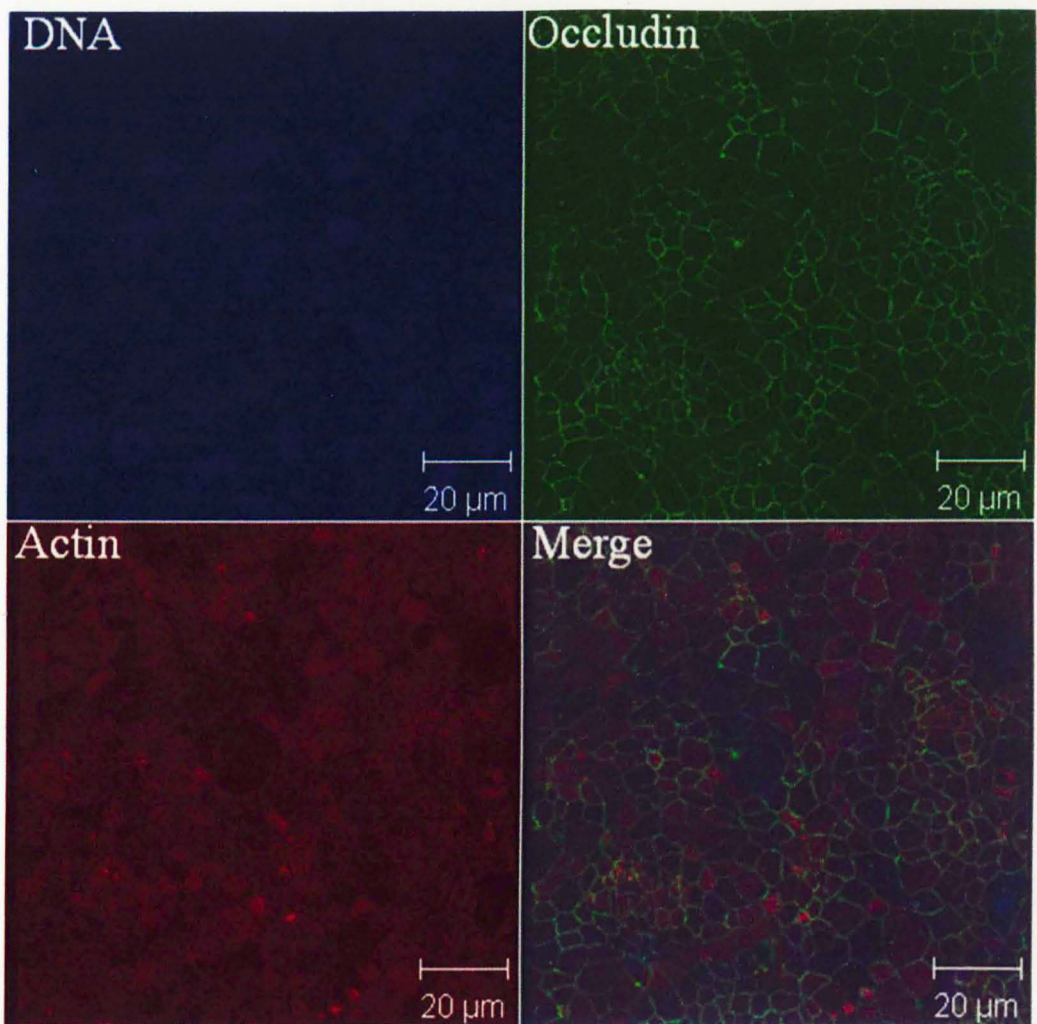


Figure 4.9: 5×10^5 T84 intestinal epithelial cells (IECs) were seeded onto Snapwell™ filters. After 14 days of growth, a filter was removed and fixed in PFA. The cells were processed for immunostaining and stained for occludin (green), actin (red) and the nuclei counterstained with DAPI (blue). The images represent projections of a stack of Z-axis slices viewed from above.

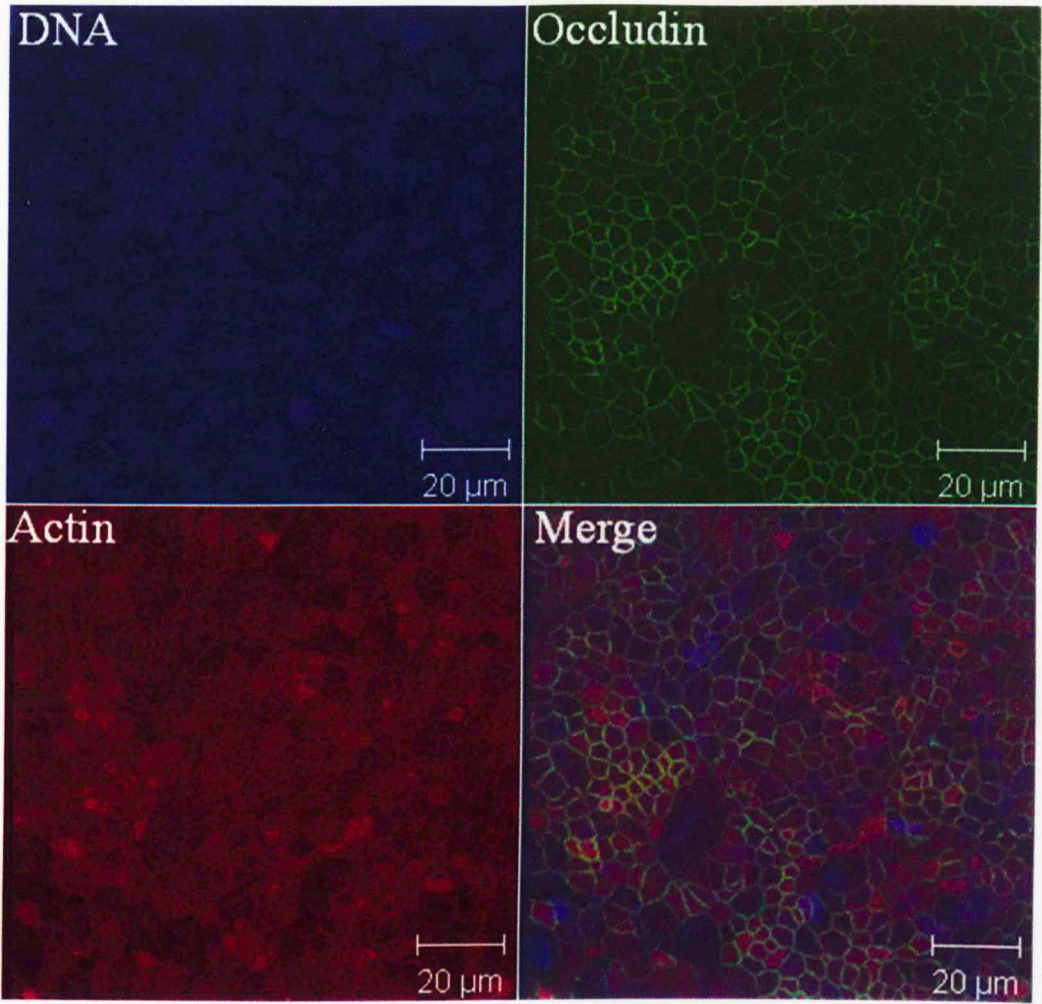


Figure 4.10: 5×10^5 T84 intestinal epithelial cells (IECs) were seeded onto Snapwell™ filters. After 21 days of growth, a filter was removed and fixed in PFA. The cells were processed for immunostaining and stained for occludin (green), actin (red) and the nuclei counterstained with DAPI (blue). The images represent projections of a stack of Z-axis slices viewed from above.

4.2.3 Analysis of the polarisation status of Caco-2 and T84 IECs growing on Snapwell™ filters over 21 days by resistance measurement indicates the formation of a polarised IEC monolayer for Caco-2 IECs

Following analysis of the permeability of Caco-2 and T84 IEC monolayers by fluorescent labelled dextran diffusion and qualitative LSCM analysis of the polarisation status, a further analysis of the IEC monolayers during the polarisation period of 21 and 14 days respectively was performed. Measuring the trans-epithelial electrical resistance (TEER) of an IEC monolayer on a permeable support is a direct, quantitative method for analysis of the polarisation status of the IEC monolayer. The TEER is a direct measurement of the ability of electrons to flow across the monolayer.

4.2.3.1 Caco-2 cells

5×10^5 Caco-2 cells were seeded directly onto the Snapwell™ filters as described in section 2.8.7. After seeding, the TEER was measured at 3 day intervals for up to 21 days using a Millicell Volt-Ohmmeter as described in section 2.8.8. When Caco-2 cells were seeded on Snapwell™ filters, the TEER increased over the test period of 21 days from $0 \Omega \text{ cm}^2$ at day 1 to $750 \Omega \text{ cm}^2$ after 21 days, confirming the formation of an impermeable, polarised monolayer (Figure 4.11).

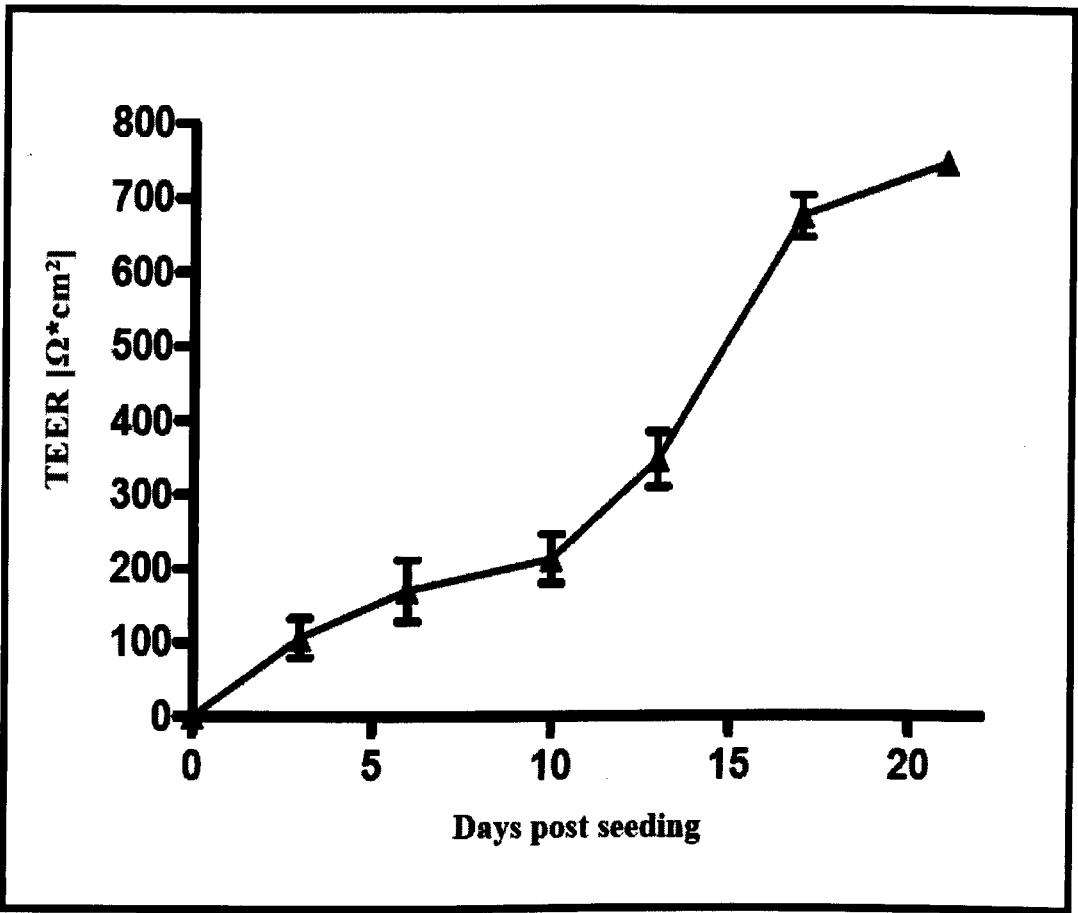


Figure 4.11: 5×10^5 Caco-2 intestinal epithelial cells were seeded onto Snapwell™ filters and the trans-epithelial electrical resistance (TEER) recorded over a 21 day period of differentiation in a humidified tissue culture incubator at 37°C. Continuous increase of the TEER suggests the development of a polarised IEC monolayer.

4.2.3.2 T84 cells

5×10^5 T84 cells were seeded directly onto the Snapwell™ filters as described in section 2.8.7 and the development of the TEER monitored over 21 days of growth. No TEER development was detectable, even after 21 days of growth. This was surprising, as both the fluorescent dextran perfusion assay and the LSCM images indicated the presence of a tight, polarised monolayer. One explanation may be the increased sensitivity of the TEER measuring method, which may be able to detect small gaps in the IEC monolayer that are neither accessible to the fluorescent dextran tracer nor identifiable by microscopy. As the T84 cells were to be used as the second cell line for confirmation of benchmark data obtained with the Caco-2 cell line, the

polarisation and impermeability of the T84 IEC monolayers after 14 days of polarisation was judged to be acceptable for the experiment. However, the issue of the lack of TEER development was considered in the interpretation of subsequent experimental data obtained with T84 IECs.

4.2.4 Ultrastructural analysis of Caco-2 cells grown on Snapwell™

filters

Whilst LSCM is sufficient to visualise the general morphology of an IEC monolayer and to highlight the distribution of certain organelles and proteins, this method does not allow visualisation of the ultrastructure of the cells. In order to investigate the ultrastructural properties of IECs grown on Snapwell™ filters, the cells were analysed by transmission electron microscopy (TEM). Analysis of 21 day old Caco-2 cells by TEM clearly demonstrated the presence of tight junctions and an apical brush border containing microvilli (Figure 4.12). This was consistent with the actin localisation as visualised by LSCM, indicating that the cells had formed a polarised monolayer.

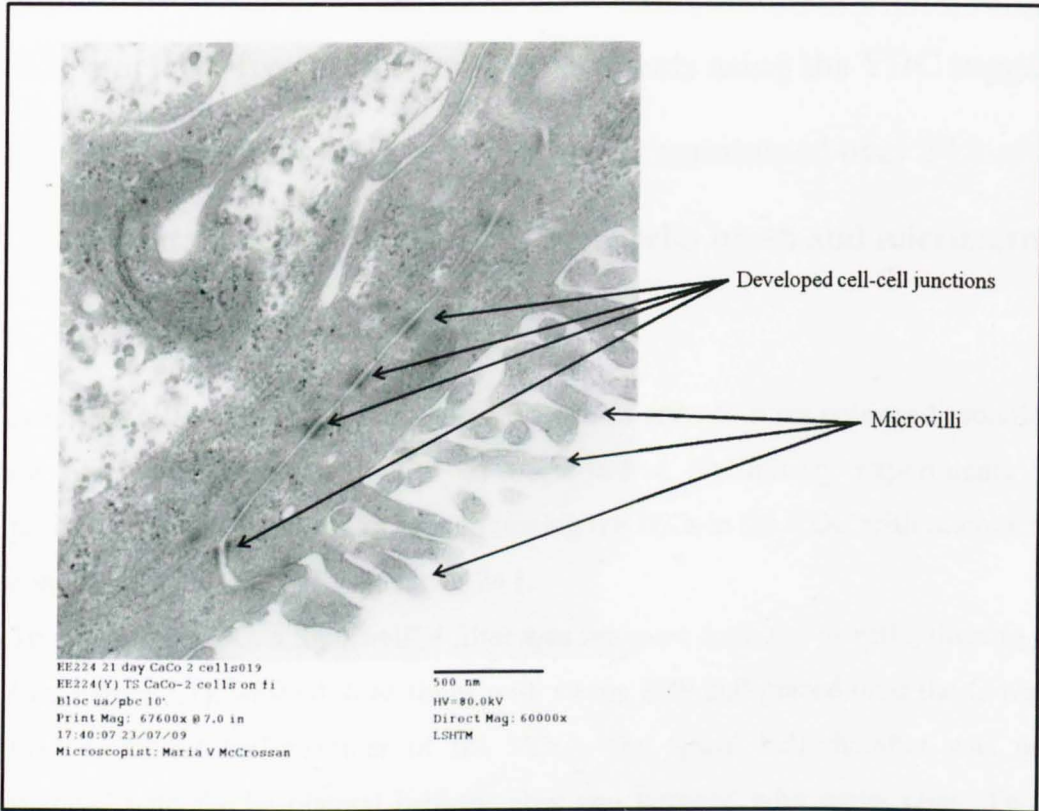


Figure 4.12: Analysis of the ultrastructural properties of Caco-2 cells that were differentiated on Snapwell™ filters for 21 days. The presence of microvilli and cell-cell junctions are indicated (arrows).

4.3 Bacteria-free preliminary experiments using the VDC suggest that the IEC monolayer integrity is maintained over 24 h of maintenance in the VDC with Brucella broth and microaerobic conditions at the apical surface

After establishing that the Caco-2 and T84 IECs grew to form polarised monolayers on Snapwell™ filters, a series of bacteria-free preliminary experiments were performed to assess the effect of maintaining the IECs in the VDC with microaerobic conditions at the apical surface over 24 h.

To set up the VDC, a Snapwell™ filter was removed from the 6-well culturing plate (see Figure 3.1), washed three times with sterile PBS and placed onto the O-ring on the basolateral half-chamber of the VDC. The apical half-chamber was gently snapped onto the basolateral half-chamber and fastened with metal clips. The two compartments were filled with the required media (Brucella broth or DMEM for the apical compartment, DMEM for the basolateral compartment), starting with the apical compartment, to avoid the possibility of liquid pressure from the basolateral compartment dislodging some of the IECs. The VDCs that were to be used for aerobic incubation experiments were placed into a humidified tissue culture incubator containing air enriched with 5% CO₂ at 37°C. The VDCs to be used for microaerobic incubation experiments were placed into the VAIN incubator and the basolateral compartment capped off, attached to the gas leads and perfused with a mixture of 95% O₂ and 5% CO₂ at a flow rate of approximately one bubble of gas every 2 to 3 seconds.

Initially both compartments of the VDC were filled with tissue culture medium, as this is the substrate in which most classical bacteria-IEC co-culturing experiments are performed. However it has been demonstrated that tissue culture medium is not optimal for *C. jejuni* culturing and that *C. jejuni* exhibits a much slower growth rate in tissue culture media than in a more suitable medium such as Brucella broth (Dr Emily Kay, personal communication). An advantage of the two-compartment setup of the VDC is the independent manipulation of the apical and the basolateral

compartments. The effects of changing media in the apical compartment from tissue culture medium to Brucella broth, whilst keeping the basolateral compartment medium as tissue culture medium was therefore also investigated.

4.3.1 Qualitative assessments of the IEC monolayer integrity after 24 h of maintenance in the VDC with microaerobic conditions at the apical surface

IECs are located at the interface between the intestinal lumen and the underlying host tissue. IECs absorb their nutrients from the basolateral membrane, whilst the apical membrane is immersed in the intestinal contents and is involved in both maintaining a barrier as well as transporting nutrients from the intestinal content into the bloodstream. The setup of the VDC, with the IECs set between the two compartments, will represent the *in vivo* situation more closely than classical *in vitro* assays. However, experimental confirmation of the integrity of the IECs in the VDC system needed to be obtained prior to performing any *C. jejuni*-IEC co-culture experiments.

During the first trial VDC experiments performed with Brucella broth in the apical compartment and tissue culture medium (DMEM) in the basolateral compartment, it was observed that even after 24 h of maintenance of a VDC with microaerobic conditions at the apical surface of the IECs, the yellow apical Brucella broth and the red basolateral DMEM remained separated. In contrast, in a VDC that had been maintained with an empty Snapwell™ filter, the two media readily diffused and mixed between the compartments (Figure 4.13). This suggests that (i): the IEC monolayer efficiently separates the two compartments even after 24 h of maintenance with microaerobic conditions at the apical surface and that (ii): Brucella broth on the apical side of the monolayer does not appear to affect the barrier function of the IECs. However, this required further quantitative assessment.

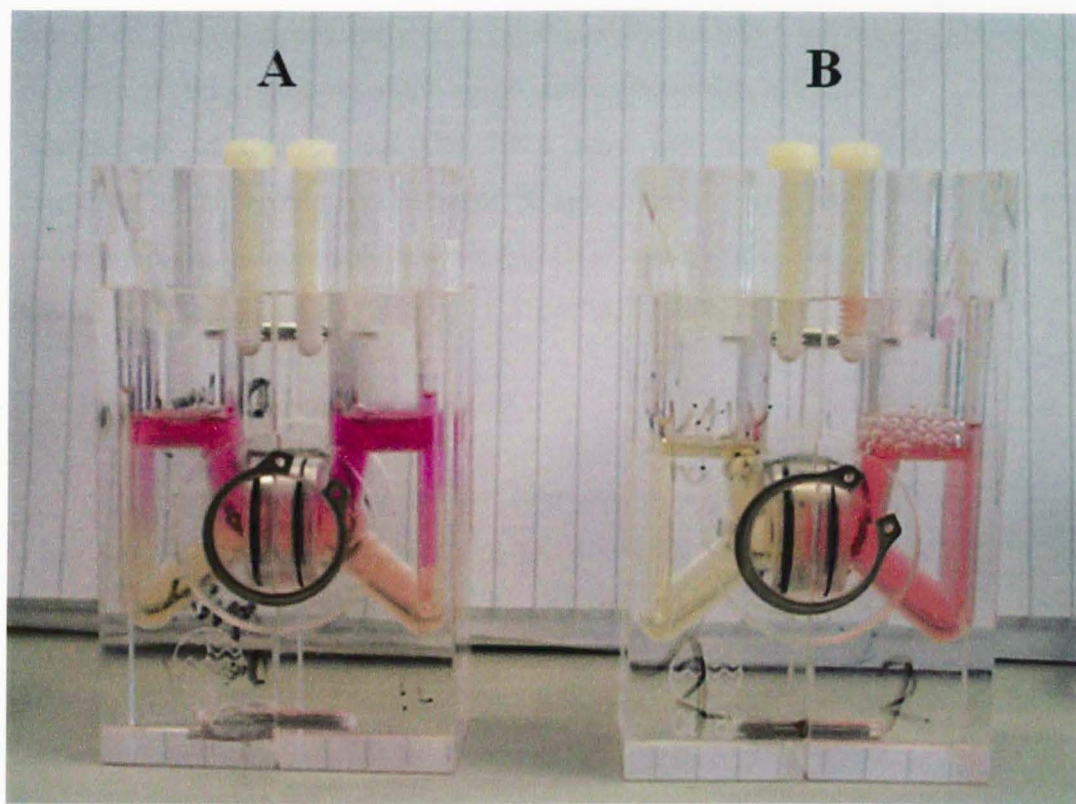


Figure 4.13: Two vertical diffusion chambers (VDCs) were set up using either an empty Snapwell™ filter (A) or using a Snapwell™ that had been used to culture Caco-2 intestinal epithelial cells (IECs) for 21 days (B). Both VDCs were maintained with microaerobic conditions at the apical surface for 24 h. Even after 24 h of such microaerobic maintenance, the IECs retained the ability to keep the two compartments separated as judged by the maintained separation of the yellow Brucella broth and the red DMEM.

4.3.2 **Assessment by fluorescently labelled dextran diffusion indicates that the IEC monolayer integrity is not affected by maintenance in the VDC for 24 h with microaerobic conditions at the apical surface**

Separation of the yellow Brucella broth in the apical compartment and the red DMEM in the basolateral compartment after 24 h of maintenance of the IECs with microaerobic conditions at the apical surface visually indicated an intact IEC monolayer. To quantitatively investigate the barrier function of the IECs after 24 h of maintenance in the VDC, the diffusion of fluorescently labelled dextran across a monolayer of both Caco-2 and T84 IECs that had been grown on Snapwell™ filters

for 21 or 14 days respectively and maintained for 24 h in VDCs with either aerobic or microaerobic conditions at the apical surface was analysed.

Monolayers of Caco-2 and T84 IECs were maintained in VDCs with either microaerobic or aerobic conditions at the apical surface for 24 h. The chambers were dismantled, the IEC monolayers washed three times with PBS and placed back into the hanging supports (see Figure 3.1). Three different controls were used:

- i: Empty Snapwell™ filters;
- ii: Snapwell™ filters that had been used to culture Caco-2 IECs for 21 days or T84 IECs for 14 days and that had been permeabilised with 0.5% (v/v) Triton X-100 in PBS at 37°C for 20 min;
- iii: Snapwell™ filters that had been used to culture Caco-2 IECs for 21 days or T84 IECs for 14 days but had not been incubated in VDCs.

500 µl of 100 µM FITC-labelled dextran with an average molecular weight of 4 kDa in Ringer's solution was added to the apical side of the monolayer and incubated for 3 hours at room temperature, with the basolateral side of the monolayer immersed in Ringer's solution. The amount of fluorescently labelled dextran on the basolateral side of the monolayer was determined post-incubation by removal of the supernatant and measurement of the fluorescence intensity at 488 nm as described in section 2.10.7. The percentage of diffused tracer was calculated from the fluorescence intensity obtained from the basolateral compartment and the fluorescence intensity of the input solution.

A Caco-2 IEC monolayer that had been grown for 21 days and not incubated in a VDC retained the fluorescently labelled dextran in the apical compartment, with only 0.5% of the fluorescently labelled dextran detected in the basolateral compartment after 3 h of incubation (Figure 4.14). In contrast, 25.2% of the fluorescently labelled dextran was detected in the basolateral compartment after 3 h of incubation using an empty Snapwell™ filter. In the case of the Triton X-100 permeabilised Caco-2 IECs, 14.3% of the fluorescently labelled dextran was detected in the basolateral compartment. For the Caco-2 IECs that had been maintained in the VDC with either aerobic or microaerobic conditions at the apical surface, low levels of fluorescently labelled dextran were detected in the basolateral compartment; 0.3% and 0.6%,

respectively. This demonstrated that maintaining the Caco-2 IECs in the VDC for 24 h with Brucella broth and either aerobic or microaerobic conditions at the apical surface did not impair the permeability of the IEC monolayer

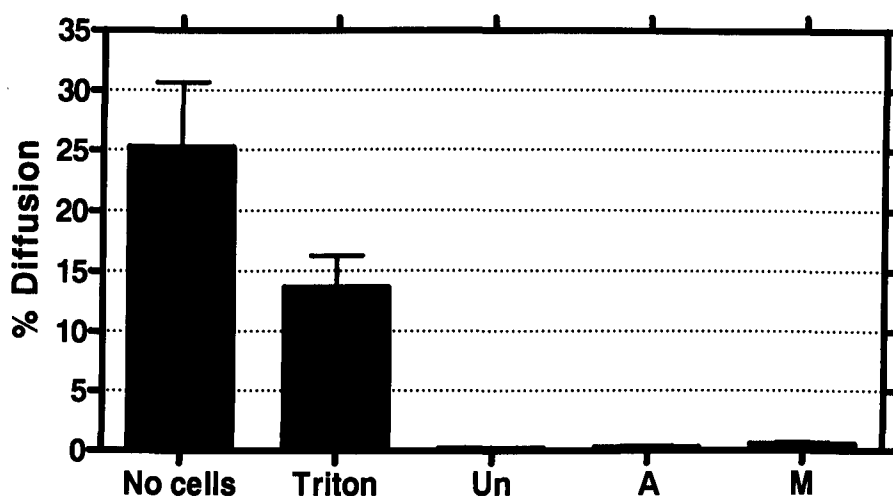


Figure 4.14: Caco-2 intestinal epithelial cells (IECs) were grown for 21 days on Snapwell™ filters and incubated in vertical diffusion chambers (VDCs) with Brucella broth and either aerobic (A) or microaerobic (M) conditions at the apical surface for 24 h. Following disassembly of the VDCs, 500 µl of 100 µM FITC-labelled dextran with an average molecular weight of 4 kilodaltons (kDa) in Ringer's solution was added to the apical side of the monolayer and incubated for 3 hours at room temperature. After 3 hours, the percentage of fluorescently labelled dextran that had passed across the monolayer was determined from the relative fluorescence of the basolateral solution at 488 nm and the relative fluorescence of the input solution. An empty Snapwell™ filter (No cells), IECs grown for 21 days on Snapwell™ filters and permeabilised with 0.5% (v/v) Triton X-100 for 20 min at room temperature (Triton) and IECs grown for 21 days on Snapwell™ filters (Un) were used as controls.

A T84 IEC monolayer that had been grown for 14 days and not incubated in a VDC successfully retained the fluorescently labelled dextran in the apical compartment, with only 0.4% of the fluorescently labelled dextran detected in the basolateral compartment after 3 h of incubation (Figure 4.15). In contrast, 25.2% of the fluorescently labelled dextran was detected in the basolateral compartment after 3 h of incubation using an empty Snapwell™ filter. In the case of the Triton X-100 permeabilised cells, 7.6% of the fluorescently labelled dextran was detected in the basolateral compartment. This was slightly lower than the number obtained for Triton X-100 permeabilised Caco-2 IECs, and might reflect a higher level detergent resistance by the T84 IECs. For the IECs that had been maintained in the VDC with either aerobic or microaerobic conditions at the apical surface, low levels of fluorescently labelled dextran was detected in the basolateral compartment; 1.4% and 1.3%, respectively. The levels were slightly higher for the T84 IECs compared to the Caco-2 IECs. This may indicate that the T84 monolayer is not as impermeable as the Caco-2 monolayer, and is in accordance with the lack of TEER found for the T84 IECs. This may also be due to the shorter growth time of the T84 IECs compared to the Caco-2 IECs (14 vs. 21 days).

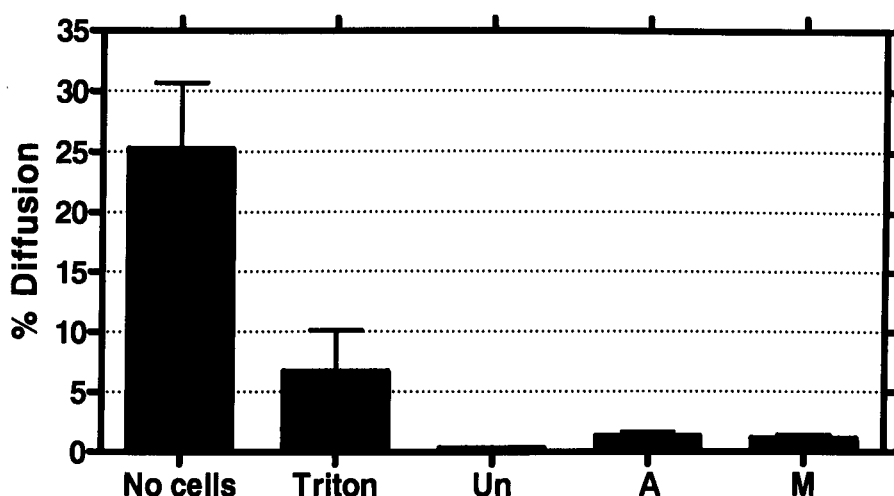


Figure 4.15: T84 intestinal epithelial cells (IECs) were grown for 14 days on Snapwell™ filters and incubated in vertical diffusion chambers (VDCs) with Brucella broth and either aerobic (A) or microaerobic (M) conditions at the apical surface for 24 h. Following the dismantling of the VDCs, 500 μ l of 100 μ M FITC-labelled dextran with an average molecular weight of 4 kilodaltons (kDa) in Ringer's solution was added to the apical side of the monolayer and incubated for 3 hours at room temperature. After 3 hours, the percentage of fluorescently labelled dextran that had passed across the monolayer was determined from the relative fluorescence of the basolateral solution at 488 nm and the relative fluorescence of the input solution. An empty Snapwell™ filter (No cells), and IECs grown for 14 days on Snapwell™ filters and permeabilised with 0.5% (v/v) Triton X-100 in PBS for 20 min at room temperature (Triton) and IECs grown for 14 days on Snapwell™ filters (Un) were used as controls.

This data suggested that maintaining the Caco-2 and T84 IECs in the VDC for 24 h with Brucella broth and either aerobic or microaerobic conditions at the apical surface did not impair the barrier function of the IEC monolayer

4.3.3 Assessment of the TEER indicates that the IEC monolayer

integrity is not affected by maintenance in the VDC for 24 h with microaerobic conditions at the apical surface

Previously TEER measurements were used to assess the polarisation status of a Caco-2 monolayer on Snapwell™ filters over a 21 day period. The same approach

was also used to quantitatively assess the effects of maintenance of the IECs in the VDC with microaerobic conditions at the apical surface on the integrity of the IEC monolayer. Initially, two VDCs were set up containing DMEM in both the apical and the basolateral compartments. The TEER was measured after the initial setup and these values set as 100%. Subsequently the VDCs were incubated with either aerobic or microaerobic conditions at the apical surface of the monolayer. The TEER was measured at 3, 6 and 24 h post setup and calculated as the percentage of the value at time point 0. Three replicate experiments were performed, and the mean and standard deviation calculated (Figure 4.16). A small and statistically non-significant decrease in the TEER was noted at 3 h post-assembly of the VDC for both conditions. However, no further decrease in TEER was noted over the rest of the 24 h maintenance period.

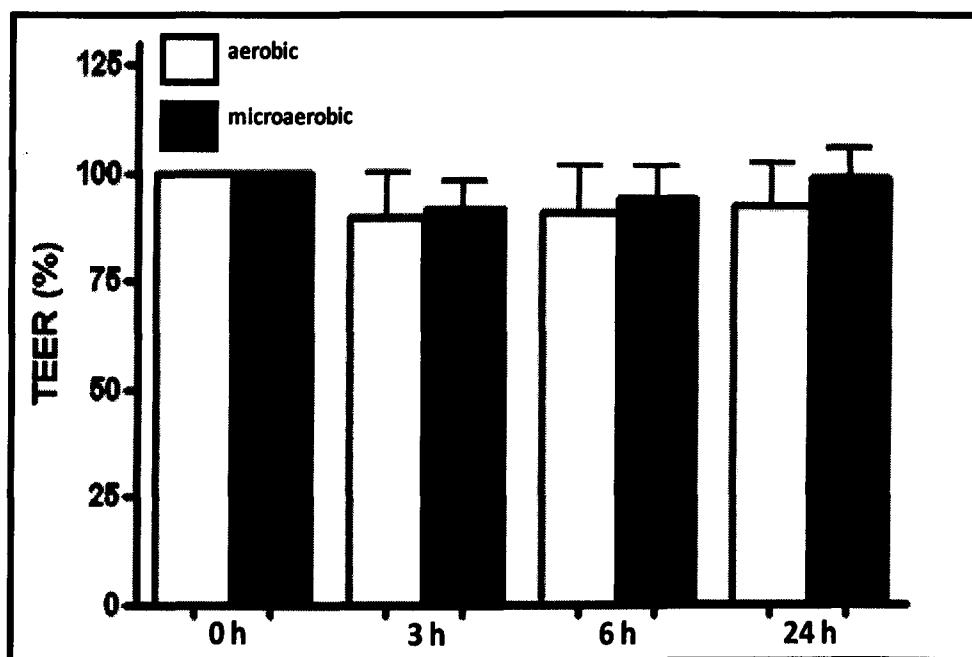


Figure 4.16: Caco-2 intestinal epithelial cells (IECs) were grown for 21 days on Snapwell™ filters and incubated in vertical diffusion chambers (VDCs) with DMEM in both compartments and either aerobic (white bars) or microaerobic (black bars) conditions at the apical surface. The trans-epithelial electrical resistance (TEER) was measured after assembly of the VDCs and set as 100% (time point 0 h). After 3 h, 6 h and 24 h post-assembly, the TEER was measured and calculated as a percentage of the value obtained at time point 0.

These experiments were then repeated with a set of VDCs where the apical compartment contained Brucella broth instead of DMEM (Figure 4.17). For the VDC maintained with aerobic conditions at the apical surface, the TEER dropped to approximately 75% of the initial value at 3 h post-assembly and remained at that level throughout the incubation. For the VDC maintained with microaerobic conditions at the apical surface, a drop to 80% of the initial value was observed 3 h post assembly. However, the TEER increased in a reproducible manner at 6 h and 24 h post-assembly, increasing to approximately 120% of the initial value after 24 hours of incubation. The difference between the TEER recorded for the two conditions reached statistical significance after 24 h of culturing in the VDC. This increase of TEER was contrary to expectations. However as the TEER was still present, this suggested that the IEC monolayer remained intact during 24 h of maintenance in the VDC with Brucella broth and either aerobic or microaerobic conditions at the apical

surface of the IECs. The increase in TEER after 24 h of culturing in the VDC with microaerobic conditions and Brucella broth at the apical surface may reflect a response of the IECs to the *in vivo* like low oxygen conditions.

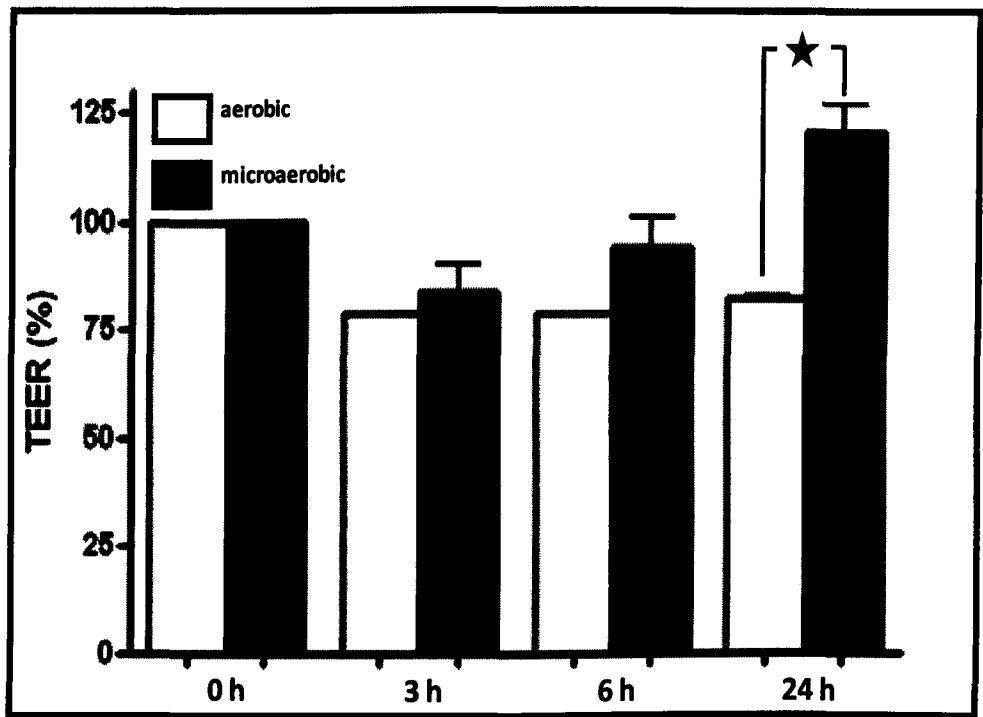


Figure 4.17: Caco-2 intestinal epithelial cells (IECs) were grown for 21 days on Snapwell™ filters and incubated in vertical diffusion chambers (VDCs) with Brucella broth in the apical compartment and DMEM in the basolateral compartment and either aerobic (white bars) or microaerobic (black bars) conditions at the apical surface. The trans-epithelial electrical resistance (TEER) was measured after assembly of the VDCs and set as 100% (time point 0). After 3h, 6h and 24 h post-assembly, the TEER was measured and calculated as a percentage of the value obtained at time point 0. * = $p < 0.05$

4.3.4 **Analysis of the distribution of cellular actin and occludin by LSCM indicates that the IEC monolayer integrity is not affected by maintenance in the VDC for 24 h with microaerobic conditions at the apical surface**

To assess the effect of maintaining a monolayer of IECs in the VDC with microaerobic conditions at the apical surface on the morphology and distribution of the tight junctions, the IECs were analysed by LSCM. Caco-2 monolayers were

grown for 21 days on Snapwell™ filters and then maintained in VDCs with Brucella broth and either aerobic (Figure 4.18) or microaerobic (Figure 4.19) conditions at the apical surface for 24 h. After incubation, the cells were prepared for LSCM as described in section 2.10.6 and stained for actin and the tight junctional protein occludin. No difference was observed regarding the distribution of actin between the IECs that had been maintained with either aerobic or microaerobic conditions on the apical surface. Furthermore, X-Z- slices of the cells demonstrated that the actin localised mainly to two areas, the cell-cell borders and the apical surface of the cells. The apical localisation of actin demonstrated that the microvilli-containing brush border remained intact, despite incubation with bacterial broth at the apical surface and had also been exposed to microaerobic conditions. No differences were observed between the distribution of occludin for IECs that had been maintained in the VDC with either aerobic or microaerobic conditions at the apical surface, indicating that the IEC monolayer remained intact and the cell-cell junctions were not affected by maintenance in the VDC for 24 h with Brucella broth and either aerobic or microaerobic conditions at the apical surface.

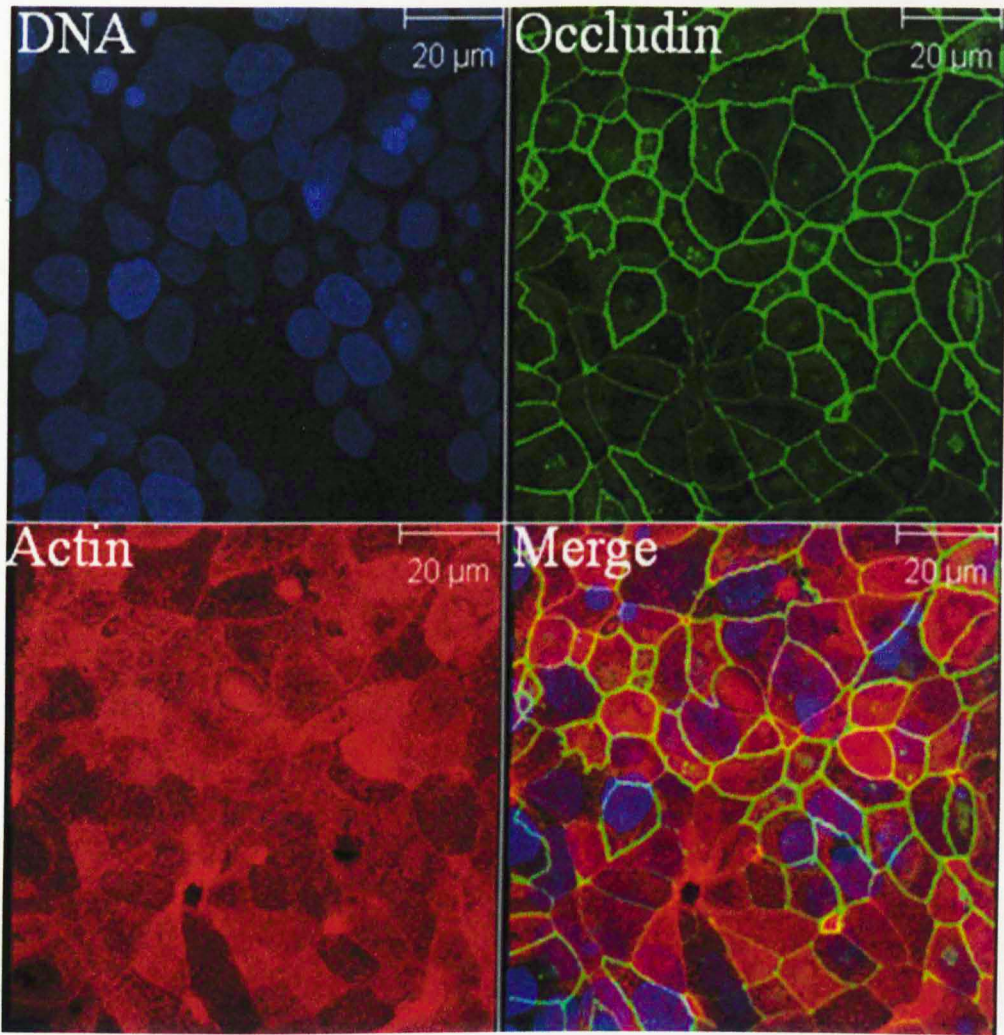


Figure 4.18: Caco-2 intestinal epithelial cells (IECs) were grown for 21 days on Snapwell™ filters and maintained in a vertical diffusion chamber (VDC) with Brucella broth in the apical compartment and aerobic conditions at the apical surface for 24 h. After the incubation, the IECs were processed for immunostaining and stained for occludin (green), actin (red) and the nuclei counterstained with DAPI (blue). The images represent projections of a stack of Z-axis slices viewed from above.

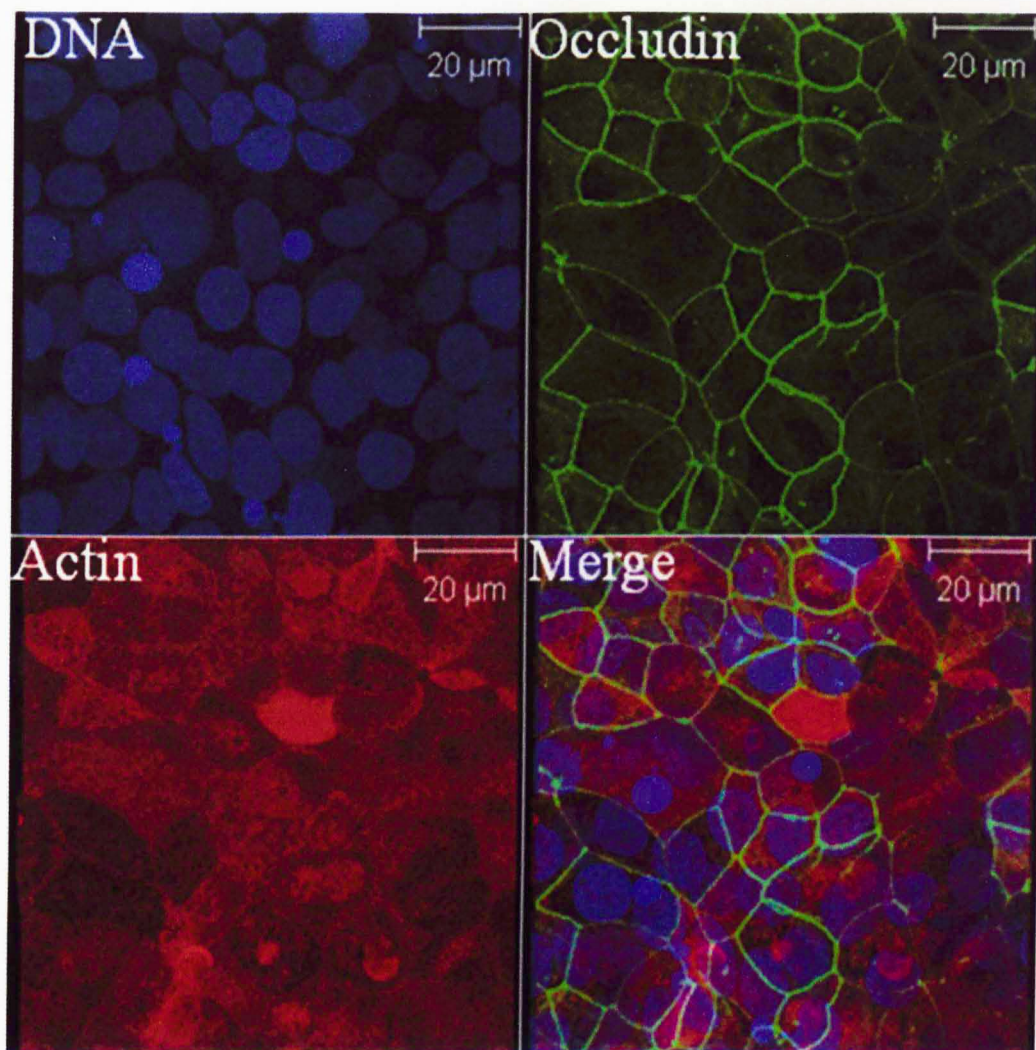


Figure 4.19: Caco-2 intestinal epithelial cells (IECs) were grown for 21 days on Snapwell™ filters and maintained in a vertical diffusion chamber (VDC) with Brucella broth in the apical compartment and microaerobic conditions at the apical surface for 24 h. After the incubation, the IECs were processed for immunostaining and stained for occludin (green), actin (red) and the nuclei counterstained with DAPI (blue). The images represent projections of a stack of Z-axis slices viewed from above.

4.4 Summary and discussion

This part of the study investigated the growth and polarisation of two human IEC lines (Caco-2 and T84) on permeable Snapwell™ filters, and assessed the effect of maintaining polarised IEC monolayers in the VDC for up to 24 h with aerobic compared to microaerobic conditions in the apical compartment. Both Caco-2 and T84 IEC lines were demonstrated to form polarised monolayers on permeable Snapwell™ filters after 21 and 14 days of growth respectively. The IECs formed

tight junctions containing occludin and display an actin-rich apical brush border. This was demonstrated by analysis of permeability of the monolayer to a fluorescently labelled tracer, by LSCM, by transmission electron microscopy and by measuring the TEER. T84 cells generally polarised faster than Caco-2 cells, with the occludin mesh appearing 7 to 14 days after seeding. Another difference observed was the absence of any measurable TEER in the T84 IECs, indicating potential gaps in the monolayers that were accessible to electrons but not to the 4 kDa fluorescent tracer. However, in light of the fact that the T84 IECs were to be used as the second confirmatory IEC line as well as for innate immune response studies, combined with the LSCM and fluorescent tracer data indicating the presence of an intact monolayer, the TEER analysis of growing T84 IECs was not further pursued. Any data obtained with the T84 IECs in further studies was analysed with consideration of the lack of TEER in the interpretation of the results.

When Caco-2 IEC monolayers were grown for 21 days on Snapwell™ filters, inserted into VDCs and maintained with Brucella broth and microaerobic conditions at the apical surface for 24 h, no apparent detrimental effects on the cells were observed compared to controls maintained with aerobic conditions at the apical surface. No increased diffusion of a fluorescent tracer was detected post-incubation. The TEER decreased to around 80% of the value at time point 0 over the first 3 h but did not decline any further. Intriguingly, the TEER increased to 120% of the value at time point 0 over 24 h when the IECs were maintained in the VDC with microaerobic conditions and Brucella broth in the apical compartment, suggesting that these conditions lead to an increase in impermeability of the IEC monolayer over time. This may reflect a direct effect of these more *in vivo* like conditions on the IECs. Analysis by LSCM indicated that the general morphology of the IECs had not changed after 24 h of maintenance with microaerobic conditions at the apical surface. The actin distribution was not affected, nor was any difference observed in the distribution of occludin.

This data suggests that the VDC model system is suitable for maintaining IECs under conditions that mimic those found in the human intestine, with the apical surface of the IECs protruding into the anaerobic environment of the gut and the basolateral surface positioned in the more favourable environment of the underlying tissue. The VDC model will thus have two benefits for the co-culturing of *C. jejuni* with IECs. Firstly, the model will more closely mimic the situation the bacteria are

likely to encounter in the human intestine. Furthermore, the co-culturing of the bacteria and the IECs will be possible under microaerobic conditions at the apical surface of the IECs, which may alter the interaction with and invasion of IECs by *C. jejuni*. This is particularly important for the microaerophilic *C. jejuni*, but may also have an effect on the pathogen-host interaction of other enteric pathogens such as the anaerobic *Clostridium difficile*. A previous study using a similar VDC setup to co-culture the microaerophilic *H. pylori* with Caco-2 IECs demonstrated increased bacterial adhesion to the cells, increased synthesis of bacterial virulence factors as well as an increased host innate immune response if the bacteria were co-cultured with the IECs in the VDC microaerobic compared to aerobic conditions in the apical compartment (Cottet *et al.*, 2002). Using the VDC model for *C. jejuni* instead of *H. pylori* has two advantages. Firstly, *C. jejuni* is able to invade IECs compared to the predominantly extracellular *H. pylori*. Secondly, the IEC lines used in this study are more closely related to the *C. jejuni* target tissue in the human host, the intestinal epithelium, compared to the gastric tissue targeted by *H. pylori*.

Chapter 5

Microaerobic conditions at the apical surface increase *C. jejuni* interactions with and invasion of intestinal epithelial cells and lead to an increased, polarised innate immune response

5.1 Introduction

The data presented in the previous chapter demonstrated that Caco-2 and T84 human IECs grow to polarised, impermeable monolayers on Snapwell™ filters over 21 and 14 days respectively. IECs could be maintained in the VDC for 24 h with Brucella broth and microaerobic conditions at the apical surface without any apparent detrimental effects on the IECs. No increase in diffusion of a fluorescent tracer molecule across Caco-2 and T84 monolayers was detected after 24 h of maintenance in the VDC with Brucella broth and microaerobic conditions at the apical surface compared to control monolayers with aerobic conditions at the apical surface. Furthermore, no significant decrease of the TEER and no difference in the distribution of cellular actin or occludin were observed after 24 h of maintenance of Caco-2 IECs in the VDC with Brucella broth and microaerobic conditions at the apical surface. The microaerobic conditions at the apical surface in the VDC more closely resemble the environment found in the intestine than the classical *in vitro* IEC-pathogen co-culturing models performed under aerobic conditions. This will allow investigation of the host-pathogen interactions under conditions more closely resembling the *in vivo* situation in the human intestine.

This chapter reports analysis of the effect of co-culturing *C. jejuni* wild-type strains with IECs maintained in the VDC with microaerobic conditions at the apical surface. *C. jejuni* wild-type strains co-cultured with IECs maintained in the VDC with aerobic conditions at the apical surface were used as controls.

5.2 Co-culturing *C. jejuni* 11168H wild-type strain bacteria with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment results in higher numbers of bacteria interacting with the IECs

The *C. jejuni* 11168H wild-type strain was co-cultured with Caco-2 IECs at an MOI of approximately 100:1 in VDCs with either aerobic or microaerobic conditions in the apical compartment for 3 h, 6 h and 24 h. The number of bacteria interacting with the IECs was determined by lysis of the IECs post co-culturing with 0.5% (v/v)

Triton-X100 in PBS for 20 min at 37°C and serial dilution and plating of the lysates as described in section 2.10.3.

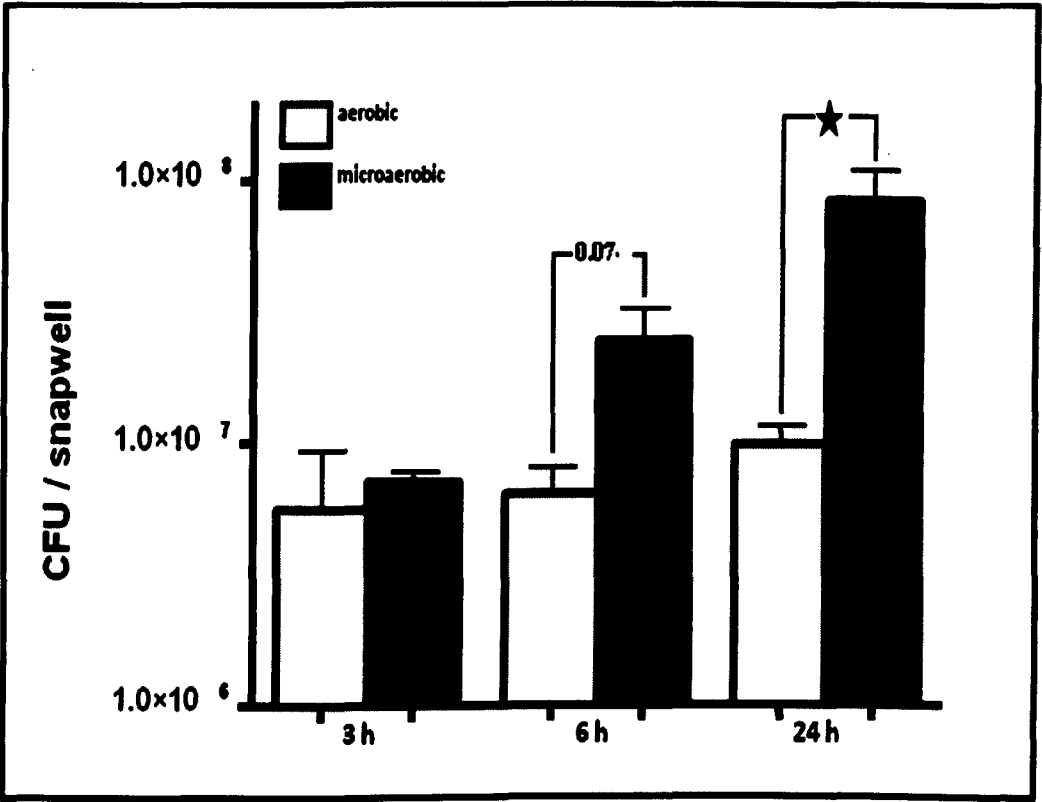


Figure 5.1: Caco-2 intestinal epithelial cell (IEC) monolayers were grown on Snapwell™ filters for 21 days. *C. jejuni* 11168H wild-type strain bacteria were co-cultured with Caco-2 IECs in a vertical diffusion chamber at an MOI of 100:1 for 3, 6 and 24 h with either aerobic (white bars) or microaerobic (black bars) conditions in the apical compartment. The numbers of interacting bacteria were determined at each time point by lysis of the IECs, serial dilution and plating of lysates. * = $p < 0.05$.

After 3 h of co-incubation, no significant difference was observed between the numbers of interacting bacteria after co-culturing with IECs in the VDC with either aerobic or microaerobic conditions in the apical compartment (Figure 5.1). Extension of the co-culturing time to 6 h resulted in increased numbers of interacting bacteria after co-culturing with the IECs in the VDC with microaerobic conditions in the apical compartment. However the difference did not reach statistical significance. Extension of the co-culturing time to 24 h resulted in a statistically significant, 8-fold increase in numbers of interacting bacteria after co-culturing with the IECs in the VDC with microaerobic conditions compared to aerobic conditions in the apical

compartment. This demonstrated that microaerobic conditions in the apical compartment of the VDC increased the numbers of *C. jejuni* interacting with the IECs.

5.3 Co-culturing *C. jejuni* 11168H wild-type strain bacteria with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment results in higher numbers of intracellular bacteria

To investigate whether the *C. jejuni* 11168H wild-type strain was also more invasive as well as more adherent when co-cultured with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment, the numbers of intracellular bacteria were determined by gentamicin protection assay as described in section 2.10.4. The 3 h timepoint was omitted, as the results from the interaction assay suggested that this co-culturing time was too short to result in any significant difference between the experiments performed with either aerobic or microaerobic conditions in the apical compartment of the VDC (Figure 5.1). *C. jejuni* 11168H wild-type strain was co-cultured with Caco-2 IECs at an MOI of approximately 100:1 in VDCs with either aerobic or microaerobic conditions in the apical compartment for 6 h and 24 h. After the co-culturing, extracellular bacteria were killed by incubation with 150 µg/ml of gentamicin in DMEM for 2 h at 37°C. The IECs were subsequently lysed, the lysates serially diluted and plated as described in section 2.10.4.

After 6 h of co-culturing, 5 times more intracellular bacteria were detected after co-culturing with IECs in the VDC with microaerobic conditions compared to aerobic conditions in the apical compartment (Figure 5.2). After 24 h of co-culturing, approximately 75 times more intracellular bacteria were detected after co-culturing with IECs in the VDC with microaerobic compared aerobic conditions in the apical compartment (Figure 5.2).

Intriguingly, the number of intracellular bacteria detected after co-culturing with the IECs in the VDC with aerobic conditions in the apical compartment dropped between 6 and 24 h, while the numbers increased after co-culturing with the IECs in the VDC with microaerobic conditions in the apical compartment. This might be an

effect of the oxygen stress encountered by the *C. jejuni* during co-culturing with the Caco-2 IECs under aerobic conditions. This data suggests co-culturing of *C. jejuni* 11168H wild-type strain with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment does not only increase the rate of bacterial interaction with the IECs, but also leads to a highly significantly increased invasion phenotype compared to co-culturing experiments with aerobic conditions in the apical compartment.

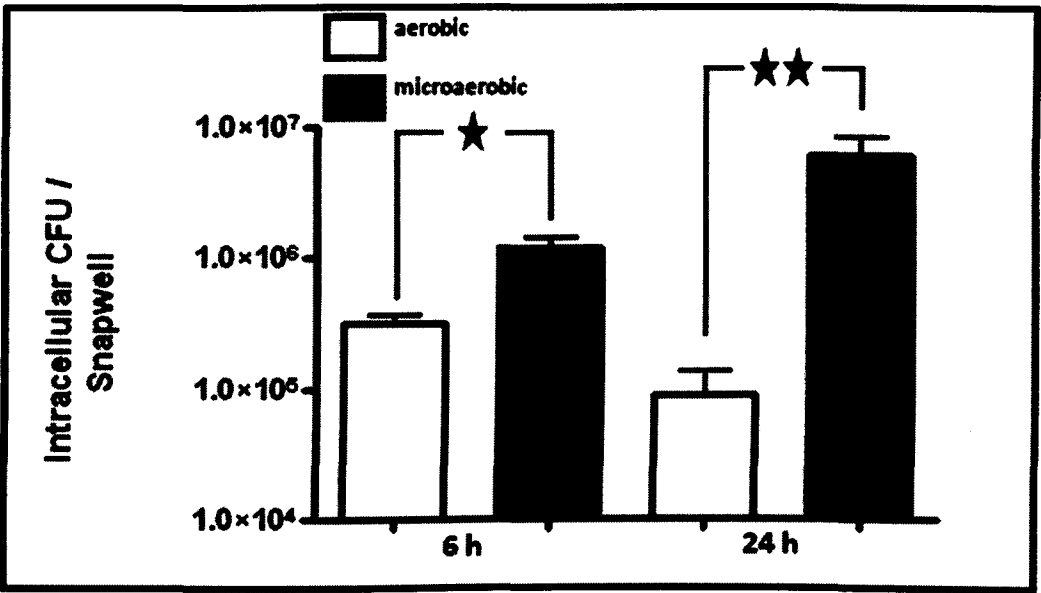


Figure 5.2: Caco-2 intestinal epithelial cell (IEC) monolayers were grown on Snapwell™ filters for 21 days. *C. jejuni* 11168H wild-type strain bacteria were co-cultured with Caco-2 IECs in a vertical diffusion chamber at an MOI of 100:1 for 6 and 24 h with either aerobic (white bars) or microaerobic (black bars) conditions in the apical compartment. The numbers of intracellular bacteria were determined at both time points by killing any extracellular bacteria with gentamicin followed by lysis of the IECs, serial dilution and plating of lysates.

* = $p < 0.05$, ** = $p < 0.01$.

5.4 Laser Scanning Confocal Microscopy supports the finding that co-culturing of *C. jejuni* 11168H wild-type strain with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment results in an increased number of interacting and intracellular bacteria

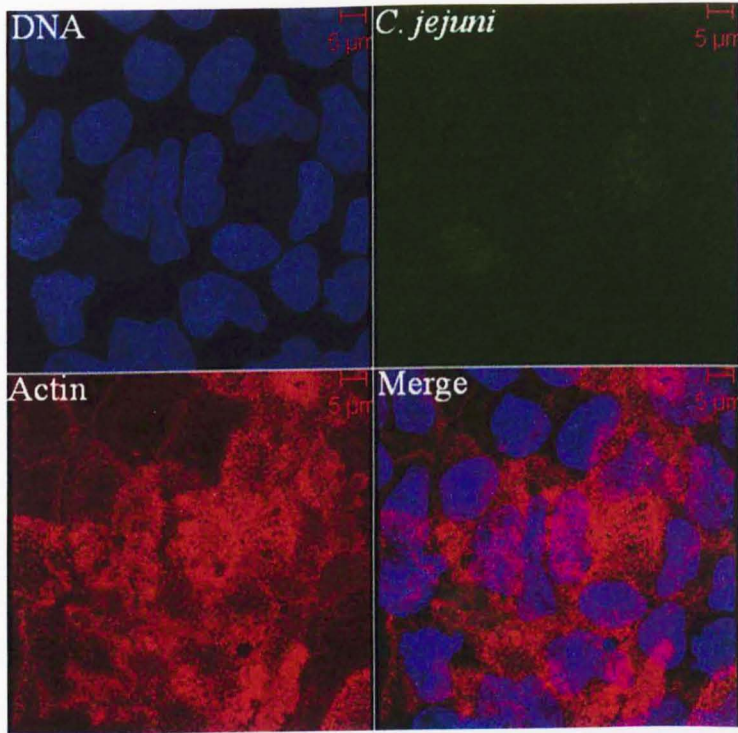
To support the quantitative data generated by the interaction and invasion assays suggesting that co-culturing of *C. jejuni* 11168H wild-type strain with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment results in an increased number of interacting and intracellular bacteria compared to control experiments with aerobic conditions in the apical compartment, a qualitative assessment of the numbers of *C. jejuni* bacteria localised within the Caco-2 IECs after 24 h of co-culturing was performed by LSCM. A *C. jejuni* 11168H wild-type strain modified to express the enhanced green-fluorescent protein (eGFP) from a chromosomal locus was utilised for this analysis (Karlyshev *et al.*, 2005b). One difficulty with expressing recombinant proteins in *C. jejuni* is the necessity to express the proteins from a chromosomal locus, as the bacteria do not stably harbour recombinant expression plasmids episomally. This reduces the number of copies of the recombinant gene to one per cell, which in turn limits the quantity of recombinant proteins produced per cell. This can be counteracted by placing the gene under the control of a strong promoter. The promoter of the outer membrane protein OmpA was used for the eGFP expressing construct used in this study. However, the fluorescence of the bacteria was still low compared to other GFP expressing bacteria such as *Yersinia enterocolitica* (Dr Philippa Strong, LSHTM, personal communication). This required the use of increased green laser power on the microscope, resulting in higher levels of green background fluorescence and rapid bleaching of the sample.

Caco-2 IEC monolayers on Snapwell™ filters were either left uninfected or co-cultured with *C. jejuni* 11168H strain expressing eGFP for 24 h in the VDC with either aerobic or microaerobic conditions in the apical compartment. The samples were prepared for LSCM as described in section 2.10.6. The IECs were

counterstained with Alexa Fluor 555 phalloidin and DAPI to visualise cellular actin and DNA as described in section 2.10.6.

No green fluorescent bacteria were detected in either of the uninfected samples (Figure 5.3). The slight hazy green background in the aerobic sample was most likely due to the increased laser power as stated previously. When the *C. jejuni* 11168H eGFP expressing strain was co-cultured with Caco-2 IECs in the VDC with aerobic conditions in the apical compartment, some green fluorescent bacterial cells were observed associated with the IECs (Figure 5.4A). However, there were only a few bacteria present in the one field of view. Furthermore, detection of bacteria was hampered by the scattering of the bacteria over several optical slices. In contrast, when *C. jejuni* 11168H eGFP expressing strain was co-cultured with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment, numerous green fluorescent bacteria were observed (Figure 5.4B), supporting the data from the interaction and invasion assays which showed that microaerobic conditions in the apical compartment of the VDC leads to higher numbers of interacting and intracellular bacteria compared to aerobic conditions in the apical compartment.

A



B

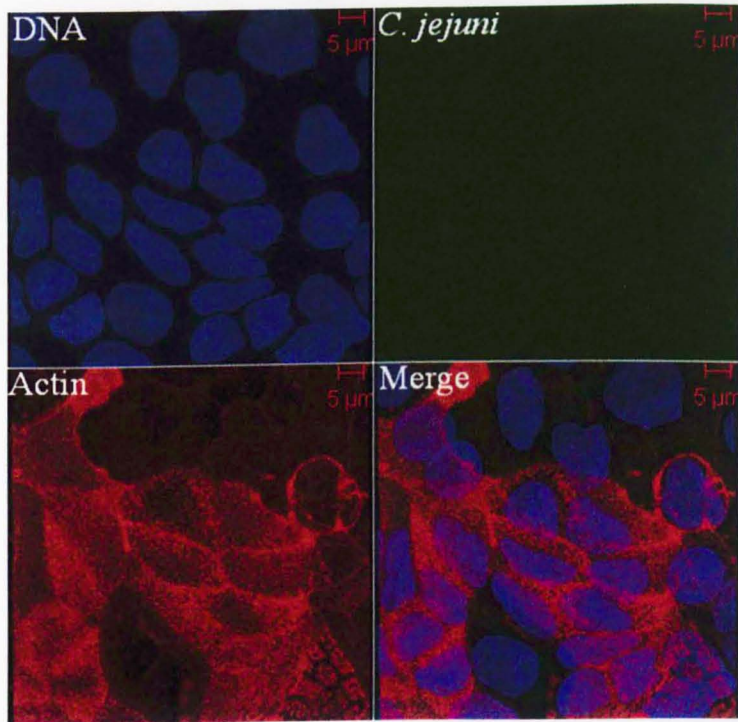
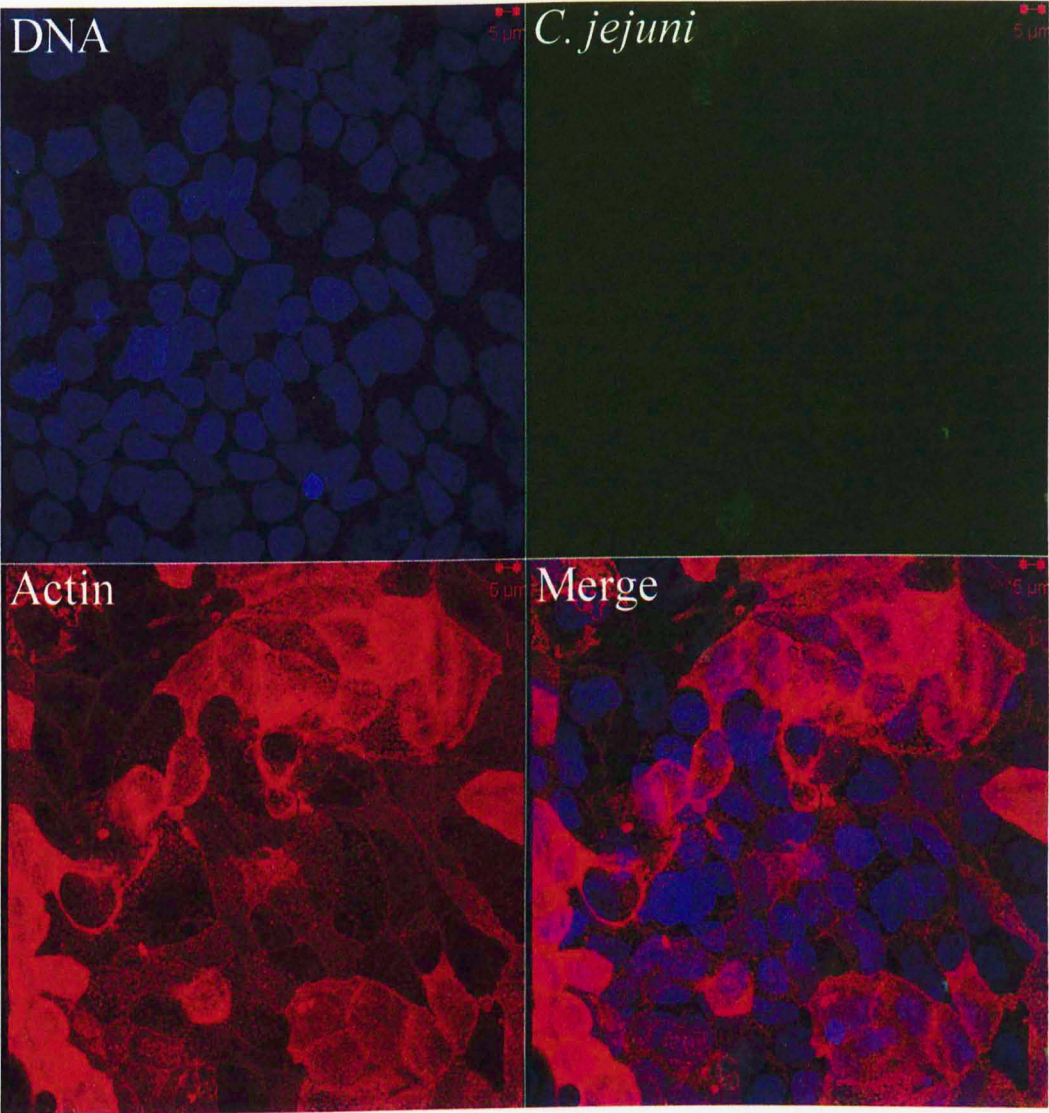


Figure 5.3: Caco-2 intestinal epithelial cell (IEC) monolayers were grown on Snapwell™ filters for 21 days. The monolayers were then maintained in a vertical diffusion chamber (VDC) for 24 h with Brucella broth and either aerobic (A) or microaerobic (B) conditions at the apical surface. The IECs were prepared for Laser Scanning Confocal Microscopy and stained for DNA (blue) and cellular actin (red).

A



B

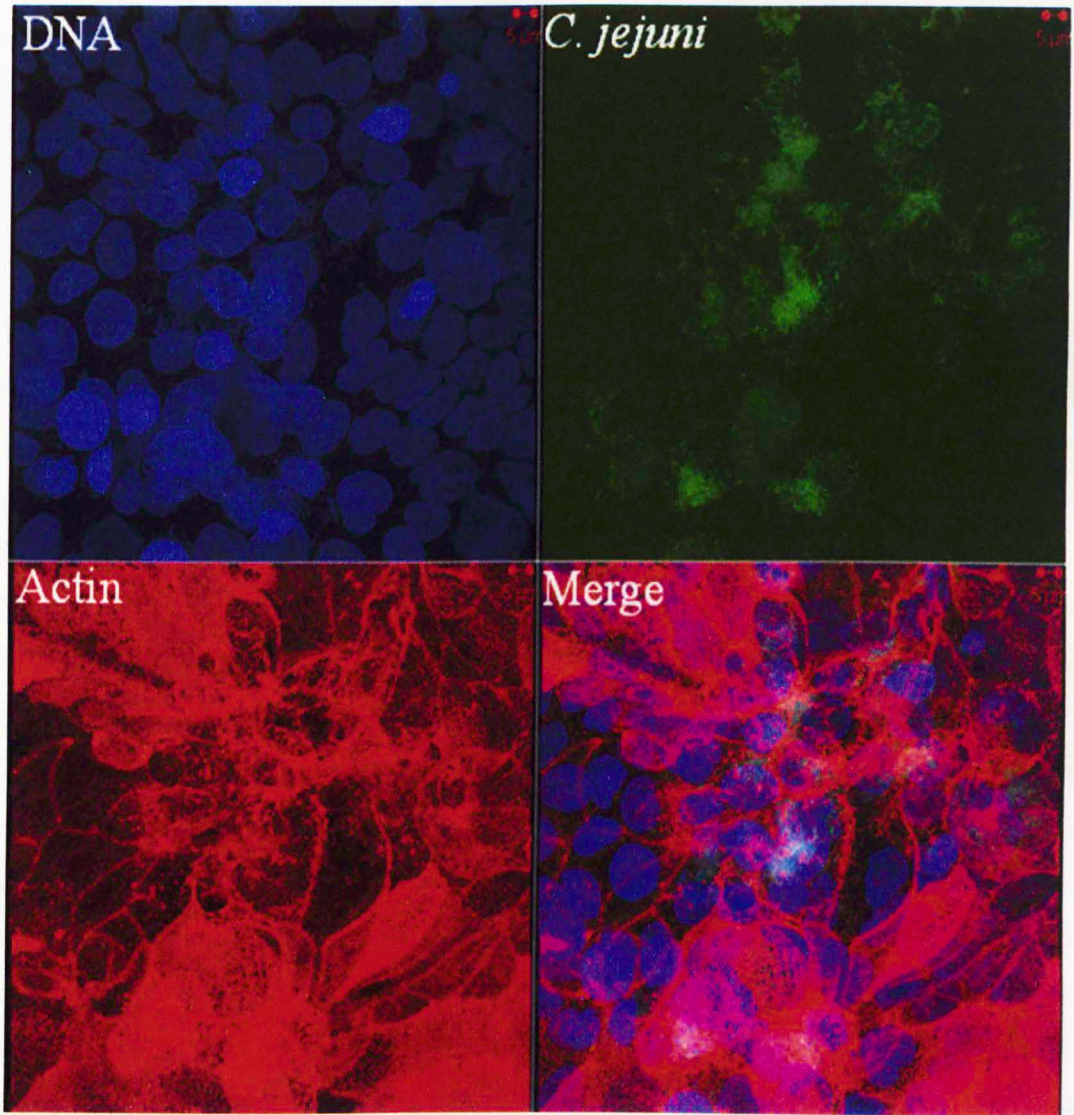


Figure 5.4: Caco-2 intestinal epithelial cell (IEC) monolayers were grown on Snapwell™ filters for 21 days. *C. jejuni* 11168H bacteria expressing eGFP were co-cultured with Caco-2 IECs in a vertical diffusion chamber (VDC) at an MOI of 100:1 for 24 h with either aerobic (A) or microaerobic (B) conditions in the apical compartment. The IECs were prepared for Laser Scanning Confocal Microscopy and stained for DNA (blue) and cellular actin (red).

5.5 Co-culturing *C. jejuni* 11168H wild-type strain bacteria with T84 IECs in the VDC with microaerobic conditions in the apical compartment for 24 h results in higher numbers of intracellular bacteria within the IECs

To eliminate the possibility of Caco-2-specific attributes leading to the increased numbers of intracellular *C. jejuni* 11168H wild-type strain bacteria after co-culturing with Caco-2 IECs in the VDC with microaerobic conditions compared to aerobic conditions in the apical compartment, these experiments were repeated with the second IEC line, T84.

The VDC model has a relatively low throughput due to i: long culturing of the IECs on Snapwell™ filters prior to the co-culturing experiments, ii: the low numbers of parallel experiments that can be performed due to the availability of only 5 VDCs and one gas manifold and iii: the long co-culturing times of up to 24 h. Consequently, only the assessment of the numbers of intracellular bacteria after 24 h of co-culturing with the IECs in the VDC with either aerobic or microaerobic conditions in the apical compartment was performed with the second cell line, as the difference between the aerobic and the microaerobic conditions was most pronounced at this time point. T84 cells were demonstrated to grow to a tight monolayer on Snapwell™ filters and no negative effects on the cells was observed during 24 h maintenance experiments in the VDC with Brucella broth and microaerobic conditions in the apical compartment (see Chapter 4). *C. jejuni* 11168H wild-type strain was co-cultured with T84 IECs at an MOI of approximately 100:1 in the VDC with either aerobic or microaerobic conditions in the apical compartment for 24 h. After the co-culturing, extracellular bacteria were killed by incubation with 150 µg/ml of gentamicin in DMEM for 2 h at 37°C. The IECs were subsequently lysed, the lysates serially diluted and plated as described in section 2.10.4 (Figure 5.5).

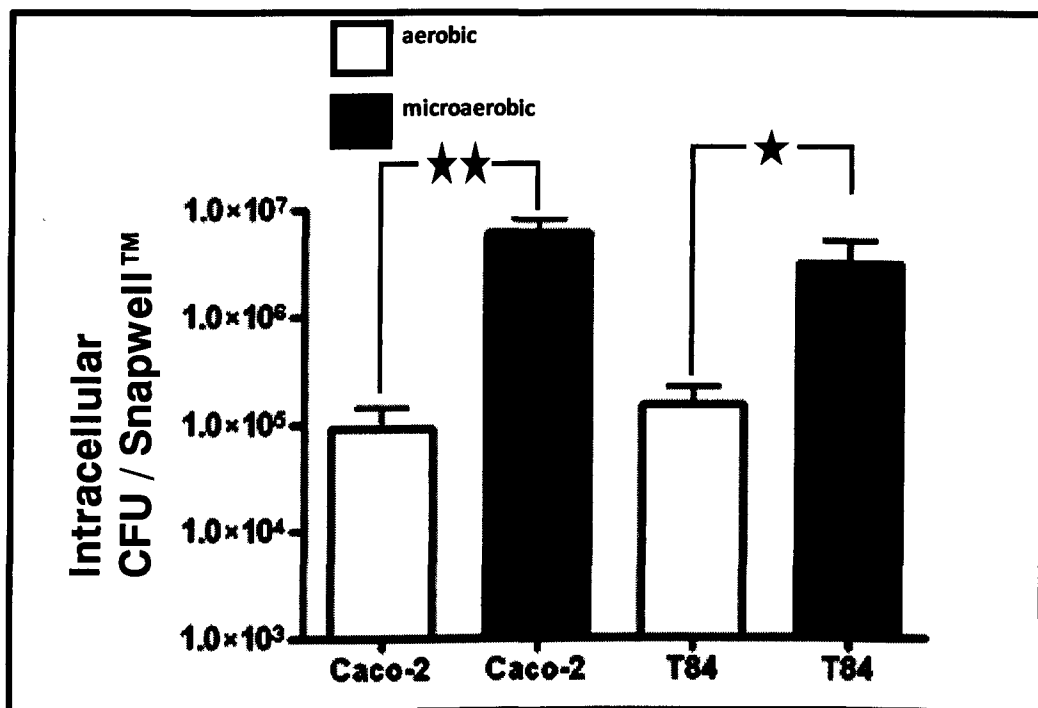


Figure 5.5: Caco-2 and T84 intestinal epithelial cell (IEC) monolayers were grown on Snapwell™ filters for 21 and 14 days respectively. *C. jejuni* 11168H wild-type strain bacteria were co-cultured with Caco-2 or T84 IECs in a vertical diffusion chamber at an MOI of 100:1 for 24 h with either aerobic (white bars) or microaerobic (black bars) conditions in the apical compartment. The numbers of intracellular bacteria were determined by killing any extracellular bacteria with gentamicin followed by lysis of the IECs, serial dilution and plating of the lysates..

As with the Caco-2 cells, co-culturing of *C. jejuni* 11168H wild-type strain with T84 IECs in the VDC with microaerobic conditions in the apical compartment resulted in more intracellular bacteria compared to co-culturing experiments performed with aerobic conditions in the apical compartment. The differences between the two conditions were not as statistically significant when using T84 IECs compared to Caco-2 cells. This might be a result of low levels of oxygen leakage across the T84 IEC monolayer due lack of complete impermeability as stated in Chapter 4. However, this data confirms the data from the Caco-2 IEC co-culturing experiments as genuine and not as an IEC line specific phenomenon.

5.6 Co-culturing *C. jejuni* 81-176 wild-type strain bacteria with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment for 24 h results in higher numbers of intracellular bacteria within the IECs

To eliminate the possibility of *C. jejuni* 11168H wild-type strain-specific characteristics leading to the observed increase in intracellular *C. jejuni* after 24 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment compared to control experiments with aerobic conditions in the apical compartment, the 24 h intracellular experiments were repeated with another widely used laboratory strain, *C. jejuni* 81-176. *C. jejuni* 81-176 wild-type strain was co-incubated with Caco-2 IECs at an MOI of approximately 100:1 in VDCs with either aerobic or microaerobic conditions in the apical compartment for 24 h. After the co-culturing, extracellular bacteria were killed by incubation with 150 µg/ml of gentamicin in DMEM for 2 h at 37°C. The IECs were subsequently lysed, the lysates serially diluted and plated as described in section 2.10.4. The numbers of intracellular bacteria obtained for *C. jejuni* 81-176 wild-type strain were comparable to the numbers obtained with *C. jejuni* 11168H wild-type strain (Figure 5.6). This suggests that the finding that co-culturing of *C. jejuni* with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment results in an increased number of intracellular bacteria compared to control experiments with aerobic conditions in the apical compartment is not strain-specific. Intriguingly, the numbers were very similar for both bacterial strains. This finding is interesting as *C. jejuni* 81-176 wild-type strain is generally considered to be more invasive than the 11168H wild-type strain. A possible explanation is that the system may be saturated after 24 h of co-culturing, and that no more bacteria are able to invade the IECs. However this is somewhat speculative. Analysis of the differences between *C. jejuni* 11168H and 81-176 wild type strains after shorter co-culturing times would shed more light on this question.

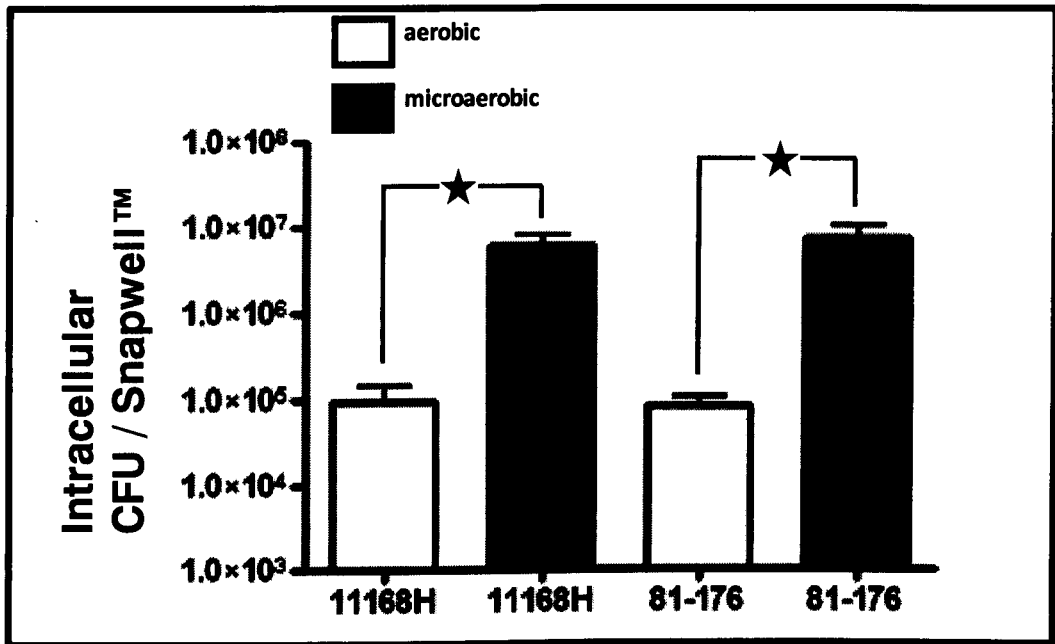


Figure 5.6: Caco-2 intestinal epithelial cell (IEC) monolayers were grown on Snapwell™ filters for 21 days. *C. jejuni* 11168H and 81-176 wild-type strain bacteria were co-cultured with Caco-2 IECs in a vertical diffusion chamber at an MOI of 100:1 for 24 h with either aerobic (white bars) or microaerobic (black bars) conditions in the apical compartment. The numbers of intracellular bacteria were determined after co-incubation by killing of any extracellular bacteria with gentamicin followed by lysis of the IECs and serial dilution and plating of the lysates. * = $p < 0.05$

5.7 The increased interaction and invasion of *C. jejuni* 11168H wild-type strain with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment results in a possible rearrangement/destruction of the occludin meshwork

After establishing that the co-culturing of *C. jejuni* 11168H wild-type strain with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment led to increased numbers of interacting and intracellular bacteria and that this was not a strain-specific result, the effect of the increased bacterial interaction and invasion on the IECs was analysed.

Previous experimental data has demonstrated that the co-culturing of IECs with *C. jejuni* leads to a disruption of the tight junctional protein occludin (MacCallum *et al.*, 2005b, Chen *et al.*, 2006). In order to investigate this in the VDC model and to

analyse whether the increased numbers of interacting and intracellular bacteria under microaerobic conditions in the apical compartment led to a change in the occludin distribution, Caco-2 IECs were co-cultured with *C. jejuni* 11168H wild-type strain in the VDC with either aerobic or microaerobic conditions in the apical compartment for 24 h (Figure 5.7B and Figure 5.8B). IECs that had been maintained in the VDC without bacteria but with either aerobic or microaerobic conditions in the apical compartment for 24 h were included as controls (Figure 5.7A and Figure 5.8A). After co-incubation, the IECs were prepared for LSCM and stained for actin and occludin as described in section 2.10.6.

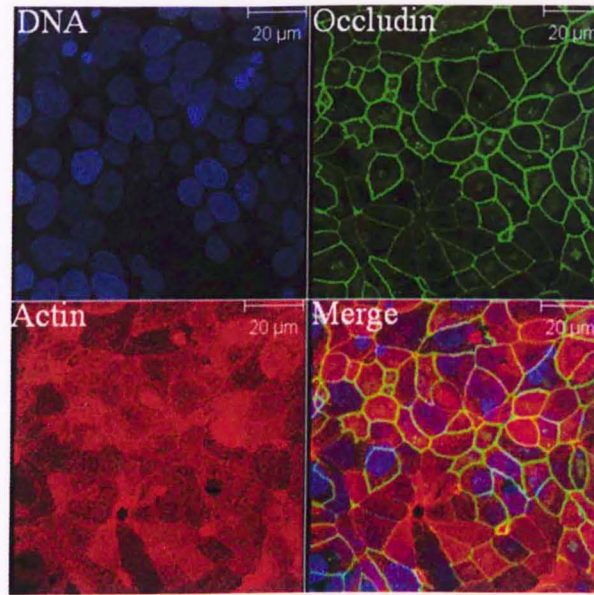
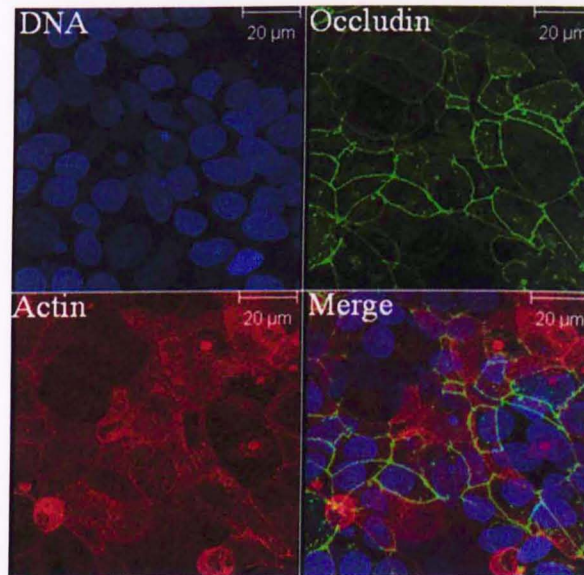
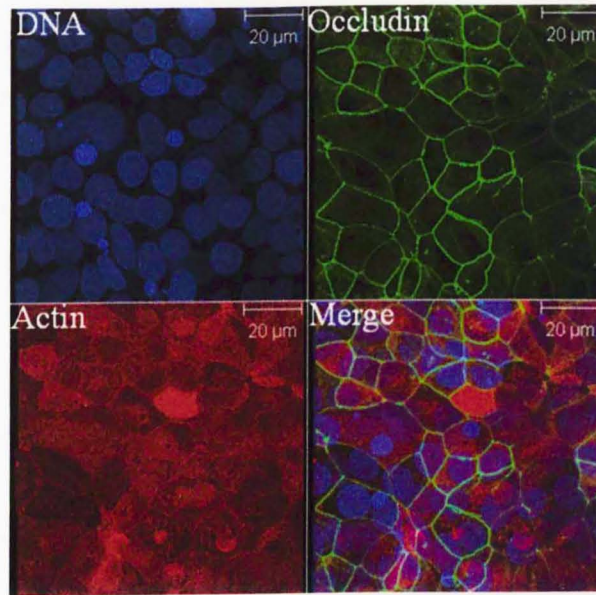
A**B**

Figure 5.7: Caco-2 intestinal epithelial cell (IEC) monolayers were grown on Snapwell™ filters for 21 days. Control IEC monolayers were maintained in a vertical diffusion chamber (VDC) with aerobic conditions, but no bacteria in the apical compartment for 24 h (A). *C. jejuni* 11168H wild-type strain bacteria were co-cultured with Caco-2 IECs in a VDC at an MOI of 100:1 for 24 h with aerobic conditions in the apical compartment. (B). After 24 h, the IECs were processed for immunostaining and stained for occludin (green), actin (red) and the nuclei counterstained with DAPI (blue). The images represent projections of a stack of Z-axis slices viewed from above.

A



B

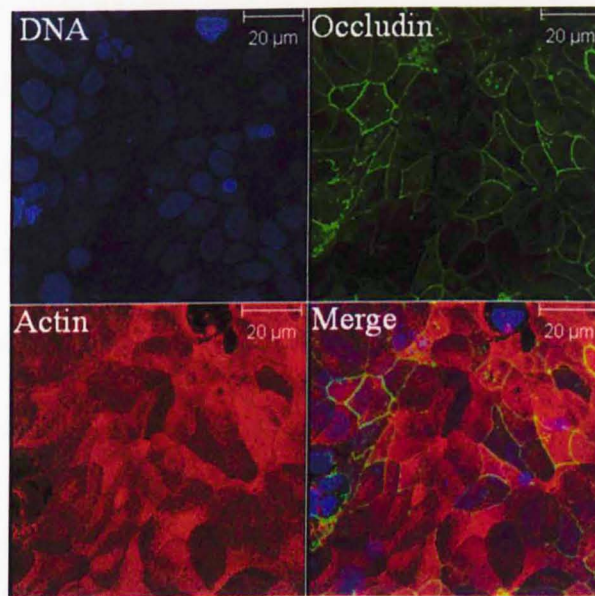


Figure 5.8: Caco-2 intestinal epithelial cell (IEC) monolayers were grown on Snapwell™ filters for 21 days. Control IEC monolayers were maintained in a vertical diffusion chamber (VDC) with microaerobic conditions but no bacteria in the apical compartment for 24 h (A). *C. jejuni* 11168H wild-type strain bacteria were co-cultured with Caco-2 IECs in a VDC at an MOI of 100:1 for 24 h with microaerobic conditions in the apical compartment. (B). After 24 h, the IECs were processed for immunostaining and stained for occludin (green), actin (red) and the nuclei counterstained with DAPI (blue). The images represent projections of a stack of Z-axis slices viewed from above.

Compared to the uninfected control samples, the occludin staining in the samples that had been co-cultured with *C. jejuni* 11168H wild-type strain appeared to be less uniform, with more areas lacking the mesh-like structure around the cells (Figure 5.7 and Figure 5.8, B compared to A). Furthermore, “blebs” of occludin that appear to have detached from the cell membranes were more frequently observed in the infected samples. However as microscopical data is not quantitative, it is difficult to conclude whether there was a significant between the aerobic and the microaerobic samples (Figure 5.7B compared to Figure 5.8B). Analysing the quantities of occludin by immunoblot using a protein unaffected by the bacterial interaction as a reference would present a more quantitative method to assess the change of occludin after 24 h of co-culturing.

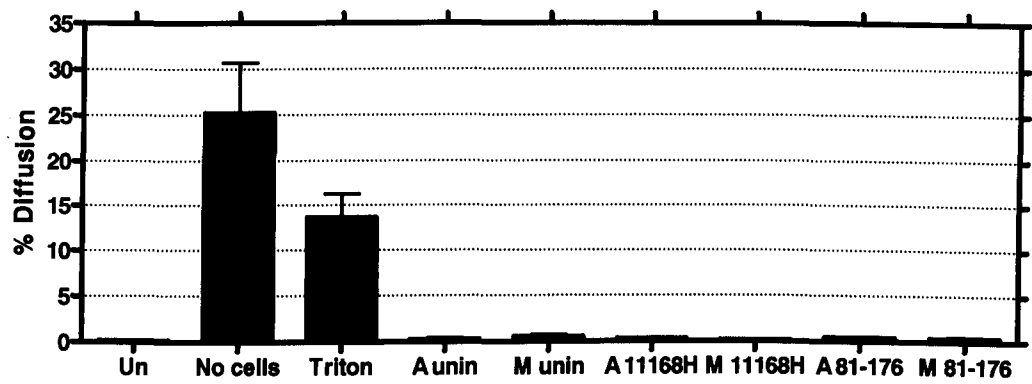
5.8 Increased interaction and invasion of *C. jejuni* 11168H and 81-176 wild-type strains co-incubated with IECs in the VDC with microaerobic conditions in the apical compartment does not result in increased permeability of the monolayer to a fluorescent tracer

As shown in section 4.1.7, the IECs grow to polarised monolayers that are impermeable to the fluorescent tracer, fluorescently labelled dextran. Evidence obtained by LSCM suggested that the distribution of occludin altered during *C. jejuni* co-culturing with the IECs, suggesting a possible change in IEC monolayer permeability. Analogous to the experiments presented in section 4.1.7, the permeability of IEC monolayers to fluorescently labelled dextran was assessed after 24 h of co-culturing with *C. jejuni* 11168H or 81-176 wild-type strains in the VDC with either aerobic or microaerobic conditions in the apical compartment. Both Caco-2 and T84 IECs were used for these experiments (Figure 5.9).

No increased permeability of the IEC monolayers to the fluorescent tracer was detected after 24 h of co-culturing of either wild-type strain with either IEC line. Furthermore, no difference was observed between either IEC line that had been co-cultured with *C. jejuni* with either aerobic or microaerobic conditions in the apical compartment, suggesting that the increased bacterial interaction did not increase

monolayer permeability after 24 h. This suggests that despite the observed change in occludin distribution (Figure 5.7 and Figure 5.8), the overall permeability of the IEC monolayer is not altered over 24 h, most likely due to other cell-cell proteins maintaining the monolayer integrity.

A:



B:

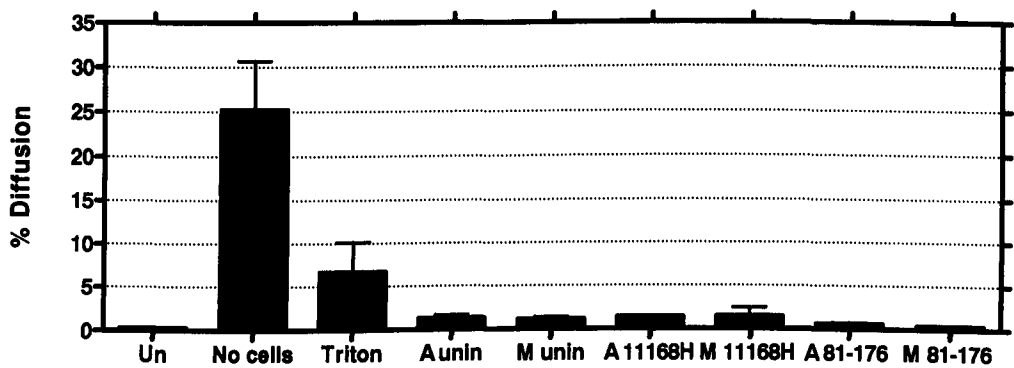


Figure 5.9: Caco-2 (A) and T84 (B) intestinal epithelial cell (IEC) monolayers were grown on Snapwell™ filters for 21 and 14 days respectively. *C. jejuni* 11168H and 81-176 wild-type strain bacteria were co-cultured with the IECs in a vertical diffusion chamber (VDC) at an MOI of 100:1 for 24 h with either aerobic (A) or microaerobic (M) conditions in the apical compartment. Untreated IECs (Un), empty Snapwell™ filters (No cells), IECs permeabilised with Triton X-100 for 20 min at 37°C (Triton) as well as IECs placed in the VDC but left uninfected with either aerobic (A unin) or microaerobic (M unin) conditions at the apical surface were used as controls. 500 µl of 100 µM FITC-labelled dextran with an average molecular weight of 4 kilodaltons (kDa) in Ringer’s solution was added to the apical side of the monolayer and incubated for 3 hours at room temperature. After 3 hours, the percentage of fluorescently labelled dextran that had passed across the monolayer was determined from the fluorescence of the basolateral solution at 488 nm and the relative fluorescence of the input solution.

5.9 The increased bacterial interactions with IECs lead to an increased, polarised innate immune response.

To assess the effect of the increased bacterial interaction with and invasion of IECs in the VDC with microaerobic conditions in the apical compartment on the innate immune response of the IECs, the VDC supernatants were analysed for the presence of markers of the innate immune system. As the IEC monolayer sits in between the two compartments of the VDC, cytokines secreted by the IECs either apically or basolaterally can be detected, allowing the detection of polarised responses.

5.9.1 The increased bacterial interactions with the IECs lead to an increased, polarised secretion of IL-8

IL-8 has been demonstrated to be secreted by IECs in response to *C. jejuni* infection *in vitro* (Hickey *et al.*, 1999, Watson *et al.*, 2005). To analyse whether the increased numbers of interacting and intracellular bacteria observed after co-culturing with IECs in the VDC with microaerobic conditions in the apical compartment were recognised by the IECs and translated into an enhanced innate immune response, the VDC co-culture supernatants were analysed by ELISA for the presence of IL-8. As the VDC is divided into an apical and a basolateral compartment, both compartments could be analysed separately, allowing for detection of a polarised, directional innate immune response. Previous published data suggested that the IL-8 response of the Caco-2 IEC line in response to *C. jejuni* infection was relatively weak under normal tissue culture conditions. Supernatants from 6 h as well as 24 h VDC co-culturing experiments with both *C. jejuni* 11168H and 81-176 wild-type strains were analysed for the presence of IL-8. Supernatants from bacteria-free VDC experiments were included as negative controls. No IL-8 was detected in any supernatants of Caco-2 IECs co-cultured with either *C. jejuni* wild-type strain for 6 h, suggesting that 6 h was not enough time to allow for the secretion of detectable amounts of the chemokine by the IECs. After 24 h of co-culturing, no IL-8 was detected in the supernatants of the uninfected controls apart from one sample (Figure 5.10). For the co-culturing experiments with *C. jejuni* 11168H wild-type strain (Figure 5.10a), IL-8 was only detected in the basolateral compartments of co-culturing experiments performed with microaerobic conditions in the apical compartment. A stronger IL-8

response was detected in the supernatants from the co-culturing experiments with *C. jejuni* 81-176 wild-type strain (Figure 5.10b). The highest levels were detected in the basolateral compartments of co-culturing experiments performed with microaerobic conditions in the apical compartment, suggesting that this condition and the resulting increased bacterial interaction and invasion leads to an increased, polarised innate immune response from the Caco-2 IECs. The finding that *C. jejuni* 81-176 wild-type strain induced a stronger IL-8 response compared to *C. jejuni* 11168H wild-type strain is particularly interesting, as the numbers of intracellular bacteria recovered after 24 h of co-culturing did not differ markedly between the two strains (Figure 5.6). This suggests that reasons other than the numbers of interacting and intracellular bacteria such as the presence or absence of certain bacterial virulence factors may lead to a differential host innate immune response.

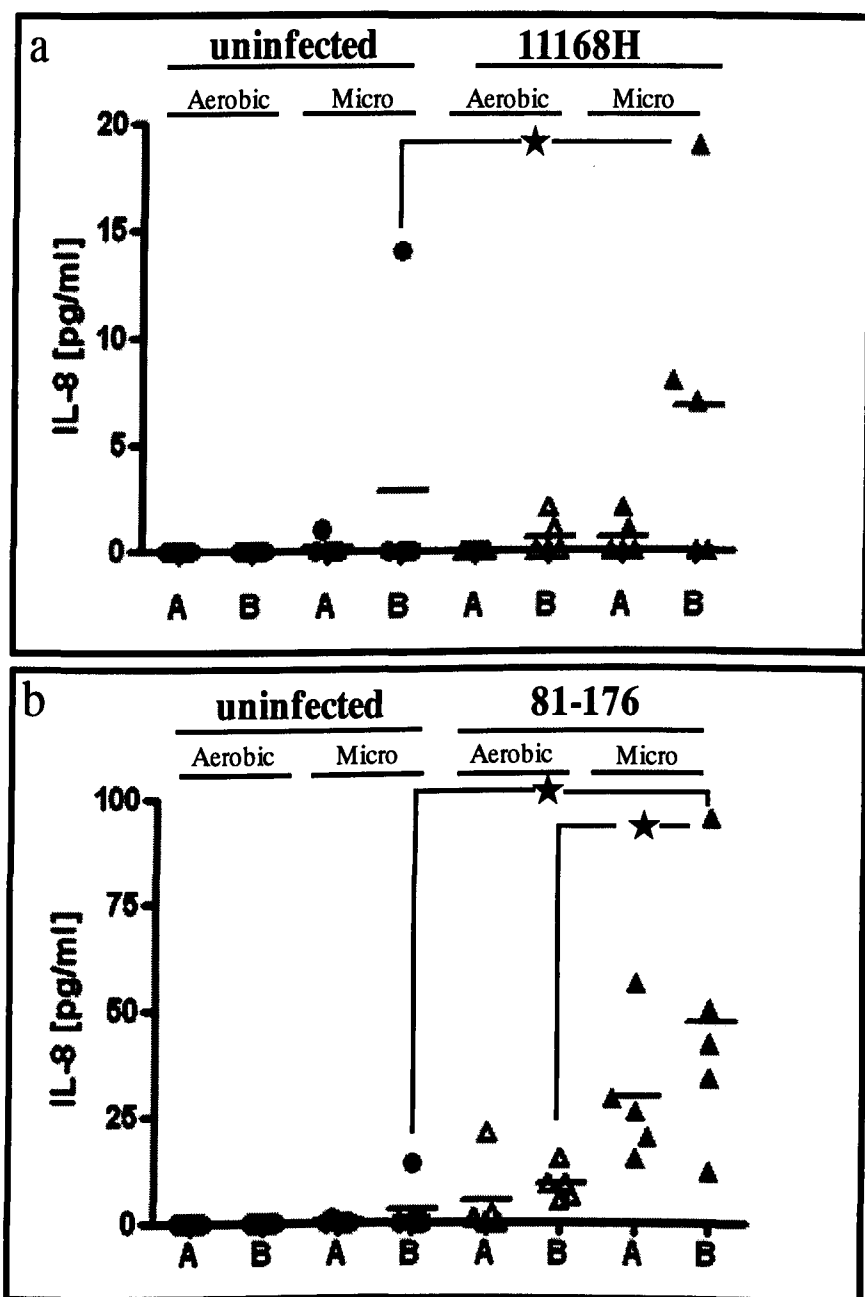


Figure 5.10: Caco-2 intestinal epithelial cell (IEC) monolayers were grown on Snapwell™ filters for 21 days. *C. jejuni* 11168H (a) and 81-176 (b) wild-type strain bacteria were co-cultured with Caco-2 IECs in a vertical diffusion chamber at an MOI of 100:1 for 24 h with either aerobic (Aerobic) or microaerobic (Micro) conditions in the apical compartment. The supernatants from the apical (A) and the basolateral (B) compartments of the VDC were probed for the presence of Interleukin 8 (IL-8) by ELISA. * = $p < 0.05$

As opposed to the relatively weak IL-8 response reported for Caco-2 IECs, T84 IECs have been demonstrated to show a more robust secretion of IL-8 in response to *C. jejuni* infection (MacCallum *et al.*, 2006). To analyse whether this higher response would be reproduced in the VDC model, supernatants of T84 cells co-cultured in the VDC with *C. jejuni* 11168H and 81-176 wild-type strains with either aerobic or microaerobic conditions in the apical compartment and the supernatants analysed by ELISA for the presence of IL-8. Supernatants from bacteria-free experiments were included as negative controls.

As opposed to the Caco-2 IECs and consistent with the literature, some IL-8 was detected in the supernatants of uninfected T84 cells (Figure 5.11). Using *C. jejuni* 11168H wild-type strain, no increase in the levels of IL-8 was detected in the supernatants from co-culturing experiments performed in the VDC with aerobic conditions in the apical compartment compared to the uninfected controls (Figure 5.11A). Furthermore, no increase in the levels of IL-8 was detected in the apical supernatants of co-culturing experiments performed in the VDC with microaerobic conditions in the apical compartment. As with Caco-2 cells, significantly more IL-8 was detected in the basolateral compartments of co-culturing experiments performed in the VDC with microaerobic conditions in the apical compartment. The levels of IL-8 detected in the basolateral compartments using T84 cells were approximately six-fold higher (average of 60 pg/ml compared to 10 pg/ml) compared to the Caco-2 cells. This confirms that, at least in response to *C. jejuni* 11168H wild-type strain, T84 IECs secrete higher levels of IL-8 than Caco-2 IECs.

Co-culturing of *C. jejuni* 81-176 wild-type strain with Caco-2 cells led to a significant increase in secretion of IL-8 by the IECs compared to IECs that had been co-cultured with *C. jejuni* 11168H wild-type strain (Figure 5.10). Intriguingly, no such difference was observed using T84 IECs and the *C. jejuni* 81-176 wild-type strain (Figure 5.11b). The highest levels of IL-8 were still detected in the basolateral compartments of co-culturing experiments performed with microaerobic conditions in the apical compartment; however, these were only slightly higher than the levels detected when *C. jejuni* 11168H wild-type strain was used (Figure 5.11). This indicates that the IL-8 response by IECs to co-culturing with *C. jejuni* is dependent on the IEC line used as well as the bacterial strain.

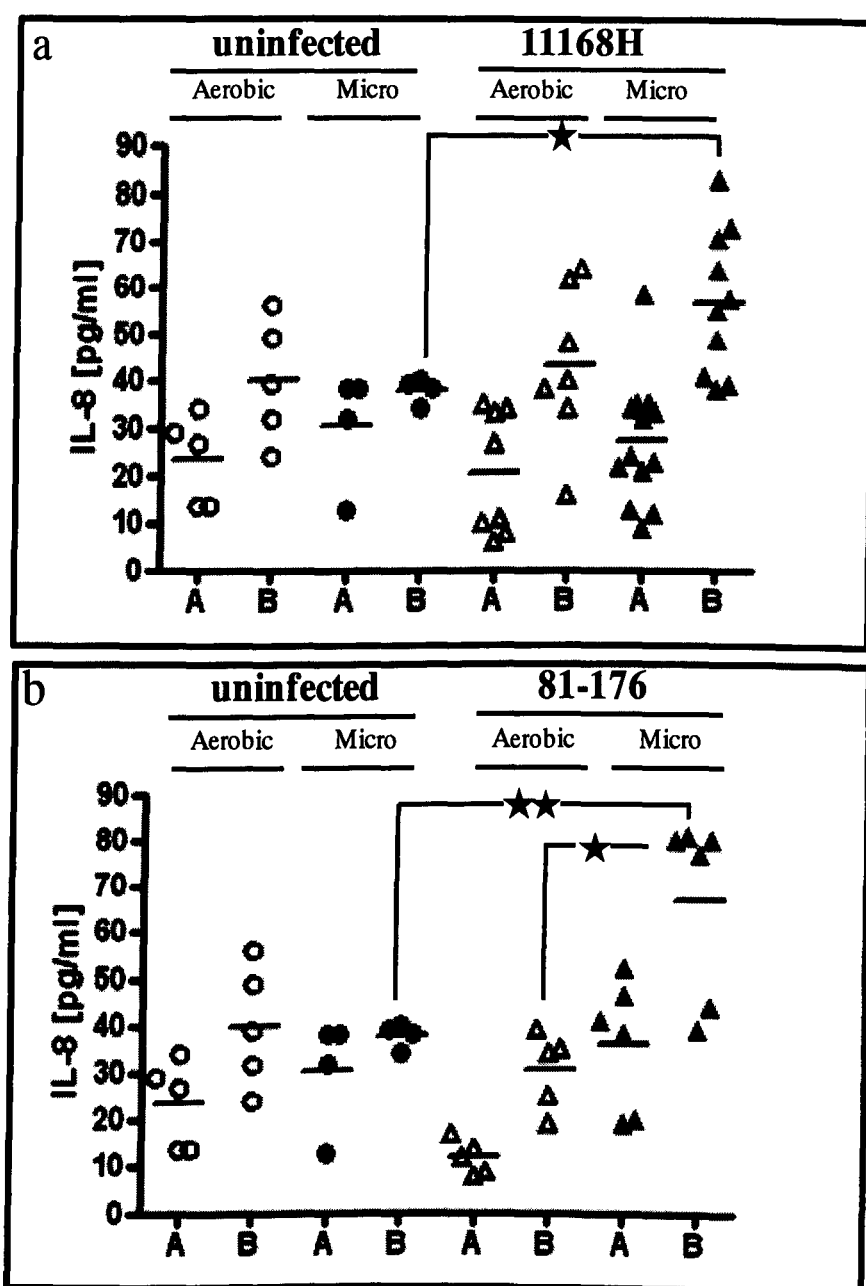


Figure 5.11: T84 intestinal epithelial cell (IEC) monolayers were grown on Snapwell™ filters for 14 days. *C. jejuni* 11168H (a) and 81-176 (b) wild-type strain bacteria were co-cultured with T84 IECs in a vertical diffusion chamber (VDC) at an MOI of 100:1 for 24 h with either aerobic (Aerobic) or microaerobic (Micro) conditions in the apical compartment. The supernatants from the apical (A) and the basolateral (B) supernatants of the VDC were analysed for the presence of Interleukin 8 (IL-8) by ELISA. * = $p < 0.05$, ** = $p < 0.01$

5.9.2 The increased bacterial interactions with the IECs lead to an increased, polarised secretion of hBD-3

Human beta defensins (hBDs) are small, antimicrobial peptides secreted in response to bacterial infection (see sections 1.3.1 and 3.4 for more details). To analyse the effect of the increased interaction with and invasion of IECs co-cultured with *C. jejuni* in the VDC with microaerobic conditions in the apical compartment on the secretion of hBDs from the IECs, the supernatants of 24 h co-culture experiments in the VDC with either aerobic or microaerobic conditions in the apical compartment were analysed by ELISA for the presence of hBD-2 and hBD-3. Supernatants from uninfected VDC experiments were included as controls. As with IL-8, both compartments of the VDC were analysed to investigate whether any response was polarised.

No hBD-2 was detected in the supernatants of any of the co-culturing experiments. As hBD-2 has previously been demonstrated to be secreted by Caco-2 IECs *in vitro* in response to co-culturing with *C. jejuni* 11168H wild-type strain, it is possible that low levels of peptide were present, but undetectable due to adherence of the peptides to the plastic tubes the supernatants had been stored in (see section 3.10)

Low levels of hBD-3 were detected in the supernatants of the uninfected co-culturing experiments (Figure 5.12). Using *C. jejuni* 11168H wild-type strain, no increased levels of hBD-3 were detected in the supernatants from co-culturing experiments performed in the VDC with aerobic conditions in the apical compartment. Low levels of hBD-3 were detected in the apical compartments of co-culturing experiments performed with microaerobic conditions in the apical compartment, but the increase was not significant compared to the uninfected controls. No increased levels of hBD-3 were detected in the basolateral supernatants from the same experiments, suggesting that the IECs secreted the hBD-3 peptides in a polarised fashion from their apical surface. Intriguingly, higher levels of hBD-3 were detected in the supernatants from the experiments using *C. jejuni* 81-176 wild-type strain (Figure 5.12b). The highest levels were detected in the apical compartments of co-culturing experiments performed with microaerobic conditions in the apical compartment, suggesting that this condition and the resulting increased bacterial interaction and invasion leads to an increased, polarised hBD-3 response from the

Caco-2 IECs. However, the low numbers of replicates limits the significance of the data, and detection of hBD-3 in the same and additional supernatants at a later time point was no longer possible, likely due to degradation of the peptides under the storage conditions used. Further investigation of the hBD-3 response of IECs co-cultured with *C. jejuni* in the VDC would be necessary, but was not carried out due to time constraints.

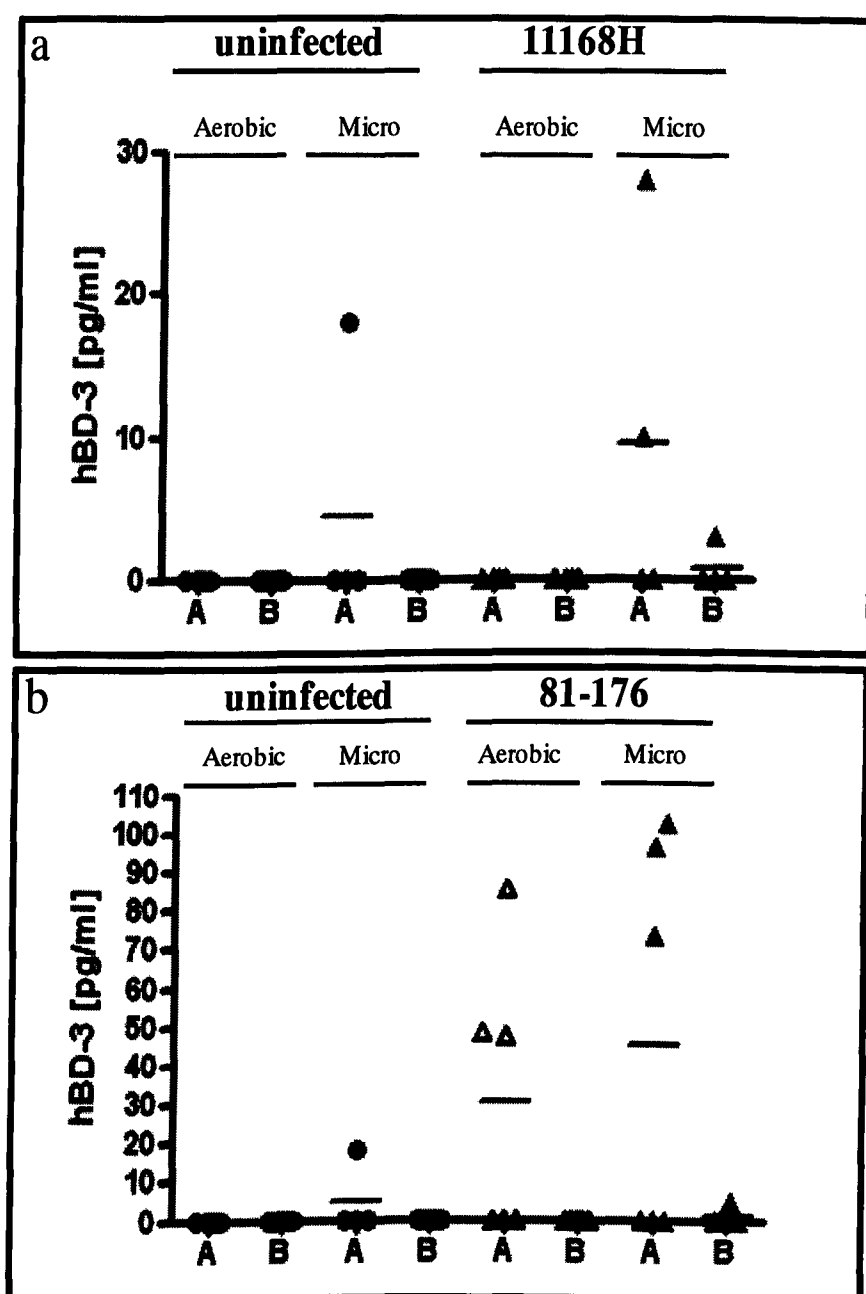


Figure 5.12: Caco-2 intestinal epithelial cell (IEC) monolayers were grown on Snapwell™ filters for 21 days. *C. jejuni* 11168H (a) and 81-176 (b) wild-type strain bacteria were co-cultured with Caco-2 IECs in a vertical diffusion chamber at an MOI of 100:1 for 24 h with either aerobic (Aerobic) or microaerobic (Micro) conditions in the apical compartment. The supernatants from the apical (A) and the basolateral (B) compartments of the VDC were probed for the presence human beta defensins 3 (hBD-3) by ELISA.

5.10 Microarray analysis of *C. jejuni* 11168H gene expression after 6 and 24 h of co-culturing with Caco-2 IECs in the VDC with either aerobic or microaerobic conditions in the apical compartment

In order to investigate the bacterial factors that contribute to the increased interaction with and invasion of IECs by *C. jejuni* after co-culturing in the VDC with microaerobic compared to aerobic conditions in the apical compartment, the gene expression profiles of *C. jejuni* 11168H wild-type strain bacteria co-cultured with Caco-2 IECs for 6 and 24 h in the VDC with either aerobic or microaerobic conditions in the apical compartment were analysed using microarrays. Total RNA was isolated from *C. jejuni* 11168H that had been co-cultured with Caco-2 IECs in the VDC for 6 h and 24 h with either aerobic or microaerobic conditions in the apical compartment as described in section 2.12. The gene expression profiles were analysed using standard microarray techniques as described in section 2.12. The exact experimental setup is shown in Figure 5.13. Replicates from three independent VDCs per condition (6 h aerobic, 6 h microaerobic, 24 h aerobic, 24 h microaerobic) were obtained. It should be noted that the RNA was isolated from bacteria that were suspended in the apical compartment and not necessarily in contact with the IECs. Hence, the data obtained will not reflect the transcription profiles of bacteria directly in contact with the IECs but the transcription profiles of bacteria under conditions leading to the observed increased interaction and invasion of IECs, which may lead to the discovery of factors involved in the observed phenotype.

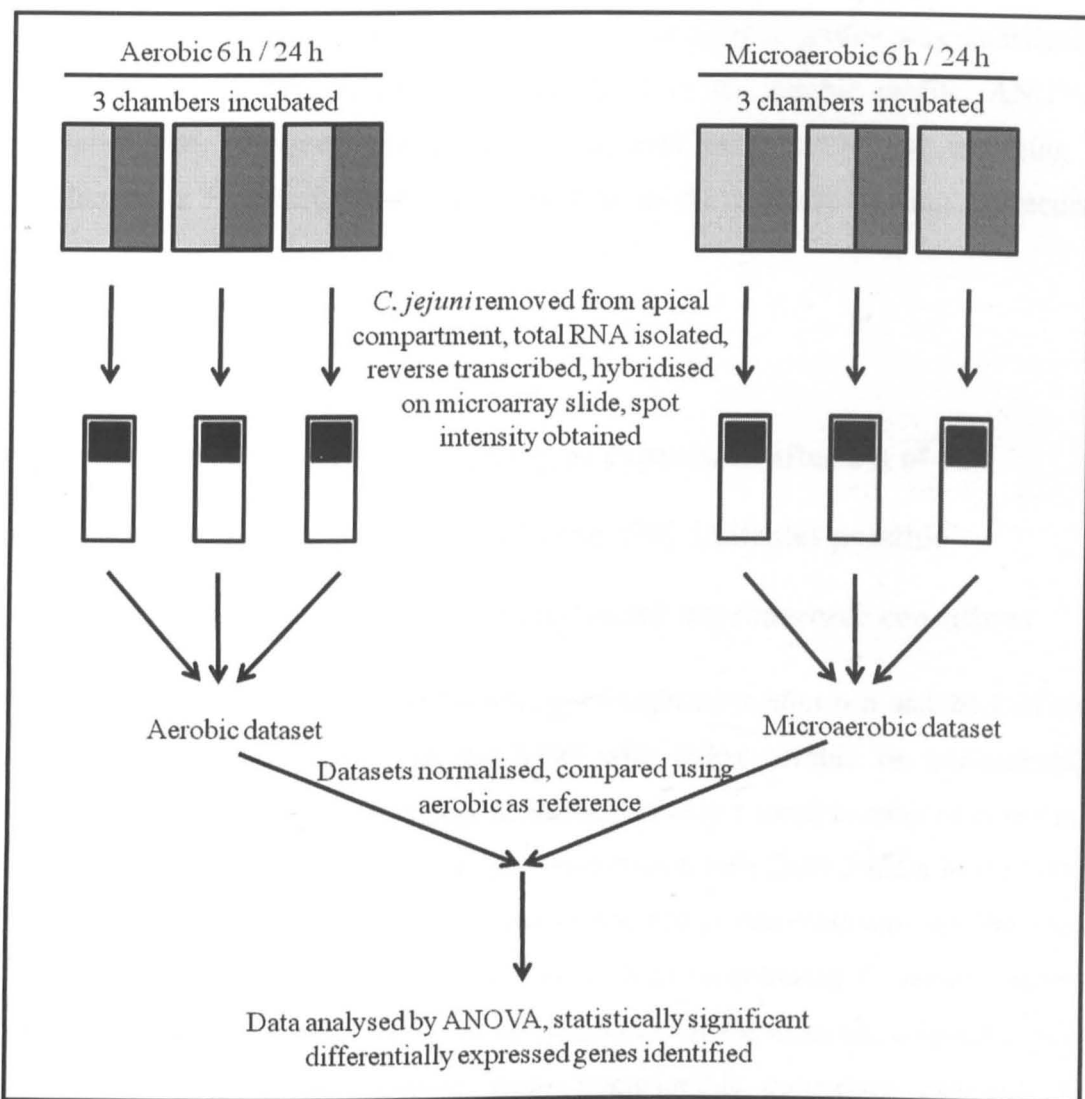


Figure 5.13: Schematic of the experimental setup of the microarray analysis

The gene expression profile for *C. jejuni* 11168H co-cultured with Caco-2 IECs with aerobic conditions in the apical compartment at each time point was set as the reference. The gene expression profile acquired for *C. jejuni* 11168H co-cultured with Caco-2 IECs with microaerobic conditions in the apical compartment was compared to the reference profile at both time points using GeneSpring software. As the classical method of gene expression analysis, where a cut-off (generally 2-fold) is chosen and the genes above and below this cut-off statistically examined for significance, yielded no statistically significant differences between the aerobic and the microaerobic gene expression profiles, an alternative method of analysis was used. The method is similar to that described previously (Kamal *et al.*, 2007). To determine which genes were differentially expressed between the aerobic and microaerobic profiles, a *P* – value of 0.05 was selected to test whether the

normalised expression level of a gene in the microaerobic profile was statistically different from the normalised expression level in the aerobic profile. ANOVA (ANalysis Of VAriance) methodology was used as the statistical test, using a Benjamini & Hochberg False Discovery Rate as the Multiple Testing Correction within the GeneSpring software.

5.10.1 Analysis of *C. jejuni* 11168H gene expression after 6 h of co-culturing with Caco-2 IECs in the VDC indicates possible differences in metabolic activity under microaerobic conditions

Microarray analysis of *C. jejuni* 11168H gene expression after 6 h and 24 h of co-culturing with Caco-2 IECs in the VDC with either aerobic or microaerobic conditions in the apical compartment indicated that only a small number of genes are differentially expressed between bacteria co-cultured with Caco-2 IECs in the VDC with aerobic or microaerobic conditions in the apical compartment. As the gene expression profiles obtained after 6 h and 24 h of co-culturing *C. jejuni* 11168H under aerobic conditions were set as the reference for the analysis, a specific gene that is indicated as up-regulated under microaerobic conditions can also be considered to be down-regulated under aerobic conditions.

5.10.1.1 *C. jejuni* 11168H genes up-regulated after 6 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment

Microarray analysis indicated that 43 genes were up-regulated in *C. jejuni* 11168H after 6 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment (selected genes Table 5.1, see Appendix I for full gene list). The gene showing the strongest up-regulation was *Cj1511c (fdhA)*, annotated as coding for a putative formate dehydrogenase large subunit protein. Formate dehydrogenase proteins (FDH) are a set of enzymes that catalyse the oxidation of formate to bicarbonate, donating the electrons to a second substrate such as

Nicotinamide Adenine Dinucleotide (NAD) (Ferry, 1990). This may be indicative of the activation of a different respiratory pathway during co-culturing under microaerobic conditions. This is supported by the finding that two other genes found to be up-regulated after co-culturing under microaerobic conditions are annotated as being involved in electron transport/respiration, *Cj1186* and *Cj0414*. Further experiments are needed to identify the role of this possible change in respiration on the interaction and invasion of the IECs. Four other up-regulated genes are annotated as coding for ribosomal proteins. This might indicate that more protein translation is occurring during microaerobic co-culturing. Furthermore, several genes coding for proteins involved in cellular metabolism were found to be up-regulated. Unfortunately no functional characterisation data for the products of these genes is available to date, making any further hypotheses regarding the involvement of these gene products in the observed increased *C. jejuni* interaction with and invasion of IECs after co-culturing in the VDC with microaerobic conditions in the apical compartment highly speculative.

One up-regulated gene that has been characterised is *dccR*. The product of this gene has been demonstrated to be the response regulator of the DccRS two-component regulatory system (MacKichan *et al.*, 2004). DccRS has been shown to be required for the *in vivo* colonisation of chicks but dispensable for *in vitro* growth, and several genes of unknown function were indicated to be regulated by the DccRS regulon (MacKichan *et al.*, 2004). A further study demonstrated activity of the DccRS regulon in the late stationary phase, identified a set of nine genes as the DccRS regulon and ruled out any influence on the transcription of genes outside the DccRS regulon (Wosten *et al.*, 2010a). Three genes within the DccRS regulon may be of particular interest; *Cj0606*, *Cj0607* and *Cj0608* have been annotated as encoding a putative type I secretion system, due to similarity to an *E. coli* alpha-haemolysin secretion system (Gundogdu *et al.*, 2007). However, none of these three genes have been functionally characterised in *C. jejuni*, so the role of *Cj0606*, *Cj0607* and *Cj0608* in host-pathogen interactions is still unclear.

Table 5.1: A selection of genes indicated by microarray analysis to be up-regulated in *C. jejuni* 11168H after 6 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment.

NCTC11168 gene number	Normalised fold-change	Annotation
<i>Cj1511c</i>	2.3	<i>fdhA</i> putative formate dehydrogenase large subunit
<i>Cj1186c</i>	2	<i>petA</i> putative ubiquinol-cytochrome C reductase iron-sulfur subunit
<i>Cj1705c</i>	2.0	Binds third domain of 23S rRNA and protein L29; part of exit tunnel
<i>Cj0414</i>	1.8	putative oxidoreductase subunit
<i>Cj1591</i>	1.6	50S ribosomal protein L36
<i>Cj0330c</i>	1.6	50S ribosomal protein L32
<i>Cj1223c (dccR)</i>	1.4	two-component response regulator
<i>Cj0961c</i>	1.4	50S ribosomal protein L34

5.10.1.2 *C. jejuni* 11168H genes down-regulated after 6 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment

Microarray analysis indicated that 24 genes were down-regulated in *C. jejuni* 11168H after 6 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment (selected gene Table 5.2, see Appendix II for full gene list).Most of these genes are annotated as putative or hypothetical, with no further information available. However one gene product has been studied in more detail. HtrB, a lipid A biosynthesis lauroyl acetyltransferase has been shown to be essential for responsiveness to harsh environments (Phongsisay *et al.*, 2007). The finding that *htrB* is down-regulated after microaerobic co-culturing (also indicating up-regulated after aerobic co-culturing) supports the hypothesis that the bacteria are under more stress during the aerobic co-culturing in the VDC.

Table 5.2: A selection of genes indicated by microarray analysis to be down-regulated in *C. jejuni* 11168H after 6 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment.

NCTC11168 gene number	Normalised fold-change	Annotation
<i>Cj1134 (htrB)</i>	1.5	lipid A biosynthesis lauroyl acyltransferase

5.10.2 Analysis of *C. jejuni* 11168H gene expression after 24 h of co-culturing with Caco-2 IECs in the VDC indicates possible down-regulation of genes involved in CPS biosynthesis and in stress response under microaerobic conditions

Microarray analysis indicated that a greater number of *C. jejuni* 11168H genes are differentially expressed between bacteria co-cultured with Caco-2 IECs under aerobic or microaerobic conditions after 24 h compared to after 6 h in the VDC.

5.10.2.1 *C. jejuni* 11168H genes up-regulated after 24 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment

Microarray analysis indicated that 73 genes were up-regulated in *C. jejuni* 11168H after 24 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment (selected genes Table 5.3, see Appendix III for full gene list). The only virulence factor identified was *ciaB* (see section 1.2.2), indicating a possible role for CiaB in mediating the increased interaction and invasion of *C. jejuni* after 24 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment. No other “classical” virulence factors were identified. Furthermore, most of the genes indicated as up-regulated under microaerobic conditions have not been functionally characterised. Perhaps the most interesting gene identified to be up-regulated is *Cj1176c (tatA)*, encoding a Sec-independent protein translocase, which is part of the Twin Arginine secretion machinery that targets proteins to the periplasm of Gram-negative bacteria (Berks,

1996). Proteins exported by this pathway include enzymes involved in electron transport. The FdhA protein found to be up-regulated after 6 h of microaerobic co-culturing (See section 5.10.1.1) has been shown to be a substrate for the TAT secretion system (Hitchcock *et al.*, 2010). Even though the *fdhA* gene was no longer up-regulated after 24 h of co-culturing under microaerobic conditions, it can be hypothesised that either i) higher levels of the protein are still present in the cytoplasm and need to be secreted or ii) the translocase may be involved in secretion of other proteins that in turn may have an effect on *C. jejuni* interactions with and invasion of IECs. However, this is highly speculative and further functional characterisation of TatA as well as construction of a 11168H *tatA* mutant and subsequent analysis of the interaction and invasion numbers would be required to support the role of TatA in *C. jejuni* interactions with IECs.

Table 5.3: A selection of genes indicated by microarray analysis to be up-regulated in *C. jejuni* 11168H after 24 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment.

NCTC11168 gene number	Normalised fold-change	Annotation
<i>Cj1176c (tatA)</i>	2.2	Sec-independent protein translocase
<i>Cj0914c (ciaB)</i>	1.3	CiaB protein

5.10.2.2 *C. jejuni* 11168H genes down-regulated after 24 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment

Microarray analysis indicated that 53 genes were down-regulated in *C. jejuni* 11168H after 24 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment (selected genes Table 5.4, see Appendix IV for full gene list). The products of three of the down-regulated genes (*Cj1447c*, *Cj1440c* and *Cj1425c*) have been demonstrated to be involved in CPS synthesis. Recent evidence demonstrated that CPS locus genes were down-regulated when the *C. jejuni* 11168H wild-type strain was co-cultured and passaged with IECs (Corcionivoschi *et al.*, 2009). It is possible that the observed down-regulation of the three genes after 24 h of co-culturing of *C. jejuni* 11168H with Caco-2 IECs in the VDC with

microaerobic conditions in the apical compartment may be a result of the co-culturing gas concentration and therefore be one of the factors involved in the increased interaction and invasion of *C. jejuni* under these conditions. Further studies using *C. jejuni* 11168H capsular mutants are needed to assess the involvement of the *C. jejuni* CPS in the increased interaction with and invasion of IECs when co-cultured with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment.

Microarray analysis indicated that four genes with annotated functions involved in DNA repair were found to be down-regulated in *C. jejuni* 11168H co-cultured with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment: *Cj0642* (*recN*), *Cj1085c* (*mfd*), *Cj0198c* (*rarA*) and *Cj0799c* (*ruvA*). This would support the hypothesis that the bacteria are experiencing more oxygen stress when co-cultured with IECs under aerobic compared to microaerobic conditions. Three flagellar genes were also down-regulated in the microaerobic dataset, *Cj0319* (*fliG*, encoding the flagellar motor switch protein G), *Cj0720c* (*fliC*) and *Cj0549* (*fliS*), indicating that altered motility under microaerobic conditions might be involved in the increased interaction with and invasion of IECs. Another gene found to be down-regulated under microaerobic conditions was *Cj0843c*, a putative lytic transglycosylase. A 11168H *Cj0843c* mutant has been shown to exhibit higher numbers of intracellular bacteria than the wild-type strain in a standard tissue culture assay (Abdi Elmi, LSHTM, personal communication). As such, it could be hypothesised that downregulation of *Cj0843c* under microaerobic conditions would lead to increased levels of interaction and invasion with the IECs. Further studies are needed to investigate the role of the *Cj0843c* protein in the *C. jejuni* IEC interactions.

Table 5.4: A selection of genes indicated by microarray analysis to be down-regulated in *C. jejuni* 11168H after 24 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment.

NCTC11168 gene number	Normalised fold-change	Annotation
<i>Cj1447c (kpsT)</i>	1.1	Capsule polysaccharide export ATP-binding protein
<i>Cj0642 (recN)</i>	1.1	Putative DNA repair protein
<i>Cj0843c</i>	1.1	Putative secreted transglycosylase
<i>Cj0319 (fliG)</i>	1.2	Flagellar motor switch protein G
<i>Cj0720c (flaC)</i>	1.2	Flagellin
<i>Cj0549 (fliS)</i>	1.3	Flagellar protein FliS
<i>Cj1686c (topA)</i>	1.4	DNA topoisomerase I
<i>Cj1085c (mfd)</i>	1.6	Transcription-repair coupling factor
<i>Cj0198c (rarA)</i>	1.8	Recombination factor protein
<i>Cj0799c (ruvA)</i>	2.9	Holliday junction DNA helicase

In summary, microarray analysis of the gene expression of *C. jejuni* 11168H after 6 h and 24 h of co-culturing with Caco-2 IECs in the VDC with either aerobic or microaerobic conditions in the apical compartment demonstrated only minor differences in gene expression between bacteria co-cultured with IECs with aerobic or microaerobic conditions in the apical compartment. Apart from *ciaB* (up-regulated after 24 h of co-culturing with microaerobic conditions in the apical compartment of the VDC), no “classical” *C. jejuni* virulence factors were identified. Additionally, genes involved in stress response and DNA damage repair were indicated to be up-regulated after co-culturing with aerobic conditions in the apical compartment. Taken together, this preliminary microarray gene expression analysis suggests that *C. jejuni* gene products that have to date not been functionally characterised might be

involved in mediating the increased interaction with and invasion of IECs observed when co-cultured in the VDC with microaerobic conditions in the apical compartment. Alternatively, the bacteria might be under more stress when co-cultured with IECs with aerobic conditions in the apical compartment of the VDC and therefore exhibit less interaction with and invasion of the IECs. However the relatively low numbers of replicates (three replicates per time point and co-culturing condition) and the fact that the mRNA isolated during these experiments most likely represents bacteria under the different co-culturing conditions rather than bacteria actually interacting with IECs render the dataset somewhat preliminary. Further replicate experiments and different RNA isolation methodology as well as follow up studies analysing the interaction and invasion ability of *C. jejuni* 11168H strains mutated in the genes identified by the microarray analysis are needed to strengthen and confirm the data obtained by this first round of experiments.

5.11 Summary and discussion

After establishing in the previous chapter that Caco-2 and T84 IECs could be maintained in the VDC with microaerobic conditions at the apical surface for at least 24 h without any apparent detrimental effects, the effect of co-culturing *C. jejuni* 11168H wild-type strain bacteria with IECs under microaerobic conditions was assessed. A time-dependent increase in the numbers of both interacting and intracellular *C. jejuni* 11168H wild-type strain bacteria was demonstrated after co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment. This was demonstrated using quantitative interaction and invasion assays as well as by semi-quantitative LSCM. The findings were further confirmed using a second *C. jejuni* wild-type strain (*C. jejuni* 81-176) as well as a second IEC line (T84) to rule out possible strain- or cell line-specific effects. Qualitative analysis of the distribution of occludin suggested a possible minor disruption of the occludin meshwork due to the increased numbers of interacting and intracellular *C. jejuni* 11168H wild-type strain bacteria after co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment. However, analysis of the diffusion of a fluorescent tracer across IEC monolayers that had been co-cultured with *C. jejuni* 11168H and 81-176 wild-type strains in the VDC did not demonstrate

increased diffusion, suggesting that the overall barrier function of the monolayer was unaffected by the increased bacterial interaction and invasion. However, the increased levels of bacterial interaction and invasion were demonstrated to lead to an increased, polarised innate immune response from the IECs. More IL-8 was detected after microaerobic co-culturing, suggesting that the IECs were able to sense and respond to the increased bacterial challenge. More IL-8 was detected in the basolateral supernatants compared to the apical supernatants. This suggests that the IL-8 secretion by the IECs occurs in a polarised fashion, with the chemokine being secreted from the basolateral side of the IECs. This concurs with the biological function of IL-8 as a neutrophil attractant, which would be of limited use in the intestinal lumen. In accordance with the literature, a marked difference in the amount of secreted IL-8 was detected between the two IEC lines used, with the T84 IECs demonstrating higher levels of secretion compared to the Caco-2 IECs. Both *C. jejuni* wild-type strains used induced similar levels of IL-8 secretion from T84 IECs, but differed in the ability to induce an IL-8 response from the Caco-2 IECs, despite demonstrating similar numbers of interacting and intracellular bacteria. This suggests that *C. jejuni* strain specific factors as well as IEC line specific factors contribute to the level of innate immune response observed in these experiments. In addition to IL-8, higher levels of hBD-3 were detected after microaerobic co-culturing of *C. jejuni* with IECs in the VDC. Unfortunately, the number of samples was too low for detailed statistical analysis. A trend for apical secretion was observed, however. This stands in contrast to the mainly basolateral secretion of IL-8, but concurs with the biological function of hBD-3 as an antimicrobial peptide. Further studies are required to clarify these observations. This data demonstrates that the two-compartmental setup of the VDC allows for efficient identification of a polarised host response.

Microarray analysis of the gene expression of *C. jejuni* 11168H wild-type strain after 6 h and 24 h co-culturing with Caco-2 IECs in the VDC with either aerobic or microaerobic conditions at the apical surface revealed relatively small differences in gene expression under the two conditions. No virulence factors were identified by this analysis, suggesting that potentially other gene products that to date have not been linked with virulence may be involved in mediating *C. jejuni*-IEC interactions. Several genes linked to stress responses were found to be up-regulated after co-culturing under aerobic conditions, suggesting that the bacteria are exposed to stress

when co-cultured with the IECs under aerobic conditions. While this finding cannot be directly linked to higher levels of interaction and invasion, it can be hypothesised that bacteria under stress will interact with IECs less readily than those under non-stressed conditions. This concurs with the finding that fewer intracellular *C. jejuni* 11168H wild-type strain bacteria were recovered after 24 h of co-culturing with Caco-2 IECs in the VDC with aerobic conditions in the apical compartment compared to the numbers obtained after 6 h of co-culturing. However genes with products putatively involved in respiration/electron transport were found to be up-regulated after co-culturing with microaerobic conditions in the apical compartment, particularly after 6 h of co-culturing. This may indicate that alternative respiratory pathways are activated during co-culturing with microaerobic conditions in the apical compartment. However the relatively low numbers of replicates (three replicates per time point and co-culturing condition) and the fact that the mRNA isolated during these experiments most likely represents bacteria under the different co-culturing conditions rather than bacteria actually interacting with IECs render the dataset somewhat preliminary. Further replicate experiments and different RNA isolation methodology as well as follow up studies analysing the interaction and invasion ability of *C. jejuni* 11168H strains mutated in the genes identified by the microarray analysis are needed to strengthen and confirm the data obtained by this first round of experiments. Further studies are required to confirm this finding. Additionally, three genes that are part of the CPS locus were found to be down-regulated after 24 h of microaerobic co-culturing. A recent study demonstrated down-regulation of CPS genes when in contact with IECs *in vitro* (Corcionivoschi *et al.*, 2009). It can be speculated that down-regulation of the CPS may lead to exposure of *C. jejuni* surface structures that may be involved in mediating bacterial interactions with the IECs. A similar mechanism has been demonstrated to occur in *Neisseria meningitidis*, where the CPS is down-regulated prior to attachment to and invasion of cells, allowing for the unmasking of surface structures involved in intimate adhesion (Deghmane *et al.*, 2002).

Gene expression was only analysed after 6 h and 24 h of co-culturing of *C. jejuni* 11168H with IECs in the VDC. These two time points offer only two snapshots of what is most likely a dynamic interaction between *C. jejuni* and the IECs over time. As changes in *C. jejuni* gene expression may occur immediately after exposure to conditions and IECs in the VDC, gene expression analysis at additional timepoints,

possibly starting as early as 30 min after co-culturing, will shed more light on the dynamics of these interactions.

As the gene expression profiles described above represent only a preliminary dataset due to the low number of replicates (three per time point and co-culturing condition, see Figure 5.13) and the fact that the mRNA isolated during these experiments most likely represents bacteria under the different co-culturing conditions rather than bacteria actually interacting with IECs, further in-depth analysis of the dataset, such as a hypergeometric analysis, to assess for over- or under-representation of a functional category of genes in any dataset was not performed. However, for future studies that will include more replicates, more time points and different RNA samples, a more in-depth analysis of the data will be appropriate to extract more information from the data

Another limitation of the microarray analysis of *C. jejuni* gene expression after co-culturing with IECs in the VDC is the relatively low yield of RNA extractable for such analysis. This is a result of the low volume of bacterial suspension as well as the low bacterial concentration used in the VDC co-culturing ($\sim 4 \times 10^8$ bacteria per VDC) compared to a standard protocol for RNA analysis, where RNA would be harvested from 2 ml of a late logarithmic phase *C. jejuni* culture containing approximately 4×10^9 bacteria (Jagannathan *et al.*, 2001, Kamal *et al.*, 2007). Pooling of samples from several rounds of co-culturing might increase the yield of mRNA. Alternatively, next generation mRNA sequencing could be used to analyse the gene expression of *C. jejuni* after co-culturing with Caco-2 IECs in the VDC. As this novel technique is becoming less expensive and has the potential for very high throughput and more quantitative analysis, this would allow for in-depth analysis of *C. jejuni* 11168H gene expression during co-culturing with Caco-2 IECs in the VDC, enabling the analysis of a greater number of time points.

The data reported in this part of the study supports the finding reported for a similar VDC model used for *H. pylori*, demonstrating increased numbers of adherent bacteria when co-cultured with Caco-2 IECs under microaerobic conditions (Cottet *et al.*, 2002). As both *H. pylori* and *C. jejuni* require microaerobic conditions for growth, the finding that microaerobic conditions promote interactions of the organisms with host cells is unsurprising. However, a recent study using a VDC to analyse the interaction of the facultative anaerobe EHEC with IECs under anaerobic conditions demonstrated increased interactions of the bacteria when co-cultured with

anaerobic/microaerobic conditions in the apical compartment of the VDC (Schuller *et al.*, 2010). This suggests that the behaviour of not only microaerobic bacteria but also of bacteria that are capable of proliferating under atmospheric oxygen concentration is changed when co-cultured with IECs under microaerobic or anaerobic conditions. This demonstrates that the VDC model is an improved and very useful model for analysis of host-pathogen interactions of a wide range of pathogenic bacteria under conditions more closely resembling the *in vivo* situation in the human intestinal lumen.

Chapter 6

Investigation of bacterial factors involved in
the increased interaction with and invasion of
IECs observed under microaerobic conditions
at the apical surface

6.1 Introduction

The data presented in the previous chapter demonstrated that co-culturing the *C. jejuni* 11168H wild-type strain with IECs in the VDC with microaerobic conditions in the apical compartment leads to an increased number of bacteria interacting with and invading the IECs. Analysis of the changes in gene expression between bacteria co-cultured with IECs with either aerobic or microaerobic conditions in the apical compartment of the VDC did not reveal any obvious virulence factors involved in the increased IEC interaction and invasion of *C. jejuni*.

As a second approach to further analyse the molecular basis of the increased bacterial interaction with and invasion of the IECs in the VDC with microaerobic conditions in the apical compartment, the numbers of interacting and intracellular bacteria were determined for three *C. jejuni* 11168H mutants. It was hypothesised that if a gene product is involved in modulating the increased interaction and invasion under microaerobic conditions, then a strain mutated in that gene would no longer exhibit the increased interaction and invasion phenotype with microaerobic conditions in the apical compartment of the VDC. This approach was successfully used to demonstrate a role for the type III secretion effector EspA in increasing the numbers of interacting EHEC with IECs. An *espA* mutant demonstrated reduced increase in interaction with the IECs when co-cultured with IECs in the VDC with anaerobic conditions in the apical compartment (Schuller *et al.*, 2010).

The three *C. jejuni* 11168H mutants chosen for this study were:

- 11168H *ciaB* mutant. The CiaB protein has been proposed to have a role in IEC invasion by *C. jejuni*. However, the exact role of the protein is controversial within the *Campylobacter* research community.
- 11168H *flaA* mutant. The major flagellin subunit protein FlaA has been demonstrated to have a role in IEC invasion by *C. jejuni*.
- 11168H *rpoN* mutant: The alternative sigma factor σ^{54} (RpoN) has been demonstrated to have a role in regulation of *C. jejuni* flagellar biosynthesis.

As the most striking differences between the numbers of interacting and intracellular bacteria were observed after 24 h when the *C. jejuni* 11168H wild-type strain was co-cultured with IECs in the VDC with microaerobic conditions in the apical compartment, the three *C. jejuni* 11168H mutants were initially assessed at this time point.

6.2 A 11168H *ciaB* mutant does not exhibit reduced numbers of interacting and intracellular bacteria when co-cultured with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment

The *Campylobacter* Invasion Antigen (Cia) proteins have been reported to be required for bacterial entry into epithelial cells (Konkel *et al.*, 1999a). It was originally proposed that the proteins were secreted by a type III secretion system (TTSS) due to sequence homology with other TTSS effector proteins (Konkel *et al.*, 1999a). However, sequencing of the first *C. jejuni* genome demonstrated the absence of a TTSS in the *C. jejuni* NCTC11168 genome (Parkhill *et al.*, 2000) and it has since been proposed that the Cia proteins are secreted via the flagellum (Konkel *et al.*, 2004). Some reports state that a *C. jejuni* F38011 *ciaB* mutant exhibits a reduction in host cell invasion and that mutation of *ciaB* abolishes secretion of all Cia proteins (Konkel *et al.*, 1999b, Konkel *et al.*, 1999a). Recently, a *C. jejuni* F38011 *ciaC* mutant was also demonstrated to exhibit reduced host cell invasion (Christensen *et al.*, 2009). However, there has always been controversy about the role of the Cia proteins in IEC invasion by *C. jejuni* and a recent report states that the invasion of a *C. jejuni* 81-176 *ciaB* mutant strain did not differ from that of the parental wild-type strain (Novik *et al.*, 2010). Data obtained at the LSHTM using a 11168H *ciaB* mutant demonstrated lower numbers of interacting and intracellular 11168H *ciaB* mutant bacteria compared to the *C. jejuni* 11168H wild-type strain using standard tissue culture assays (Neveda Naz, LSHTM, personal communication). However, this data has not yet been confirmed using a complemented 11168H *ciaB* mutant.

The decision was made to assess the interaction and invasion numbers of the 11168H *ciaB* mutant in the VDC model.

A 11168H *ciaB* mutant was obtained from Neveda Naz (LSHTM) and insertion of the Kan^R cassette into the *ciaB* gene verified by PCR (Figure 6.1).

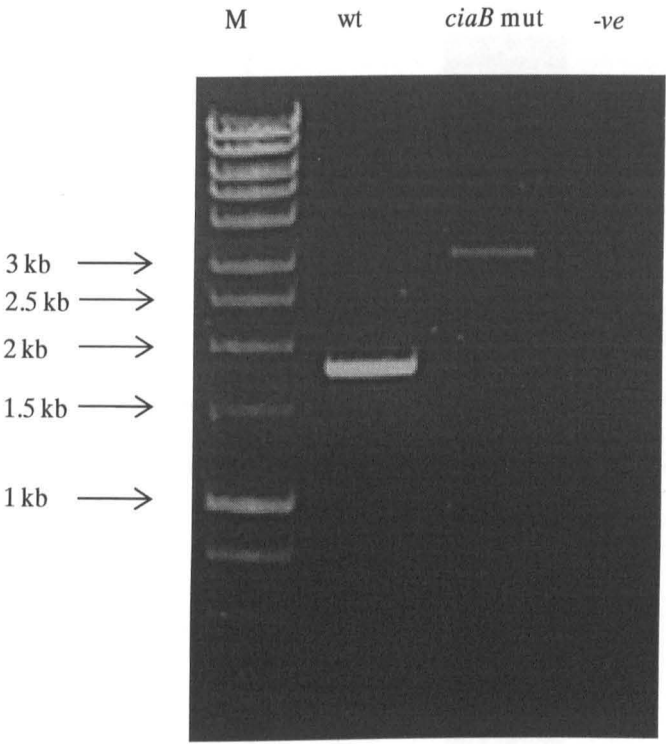
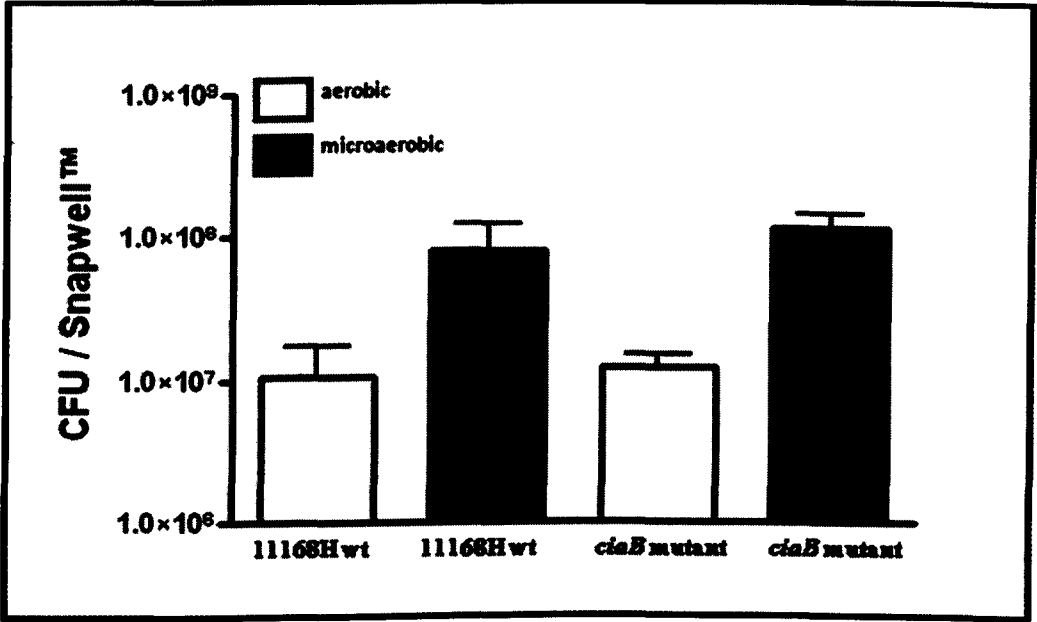


Figure 6.1: Genomic DNA was isolated from the *C. jejuni* 11168H wild-type strain and 11168H *ciaB* mutant. Primers annealing to the 5'- and the 3'-end of the *ciaB* gene were used to amplify PCR products of 1.8 kb and 3.2 kb from the wild-type strain and mutant genomic DNA templates respectively, demonstrating successful disruption of the *ciaB* gene in the 11168H *ciaB* mutant.

After verifying mutation of the *ciaB* gene, the 11168H *ciaB* mutant was co-cultured with Caco-2 IECs in the VDC for 24 h with either aerobic or microaerobic conditions in the apical compartment and the numbers of interacting bacteria determined by lysis of the IECs post incubation and serial dilution and plating of the lysates (Figure 6.2A). The numbers of intracellular bacteria were determined by killing the extracellular bacteria with gentamicin prior to IEC lysis as described in section 2.10.4 (Figure 6.2B). The numbers obtained were compared to those previously obtained with the *C. jejuni* 11168H wild-type strain (See sections 5.2 and 5.3).

A



B

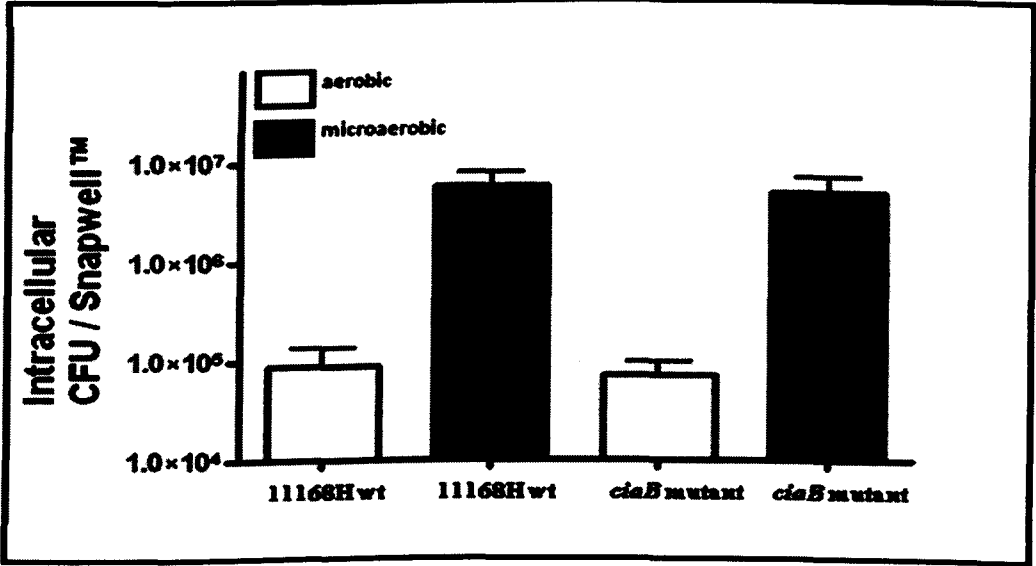


Figure 6.2: *C. jejuni* 11168H wild-type strain and the 11168H *ciaB* mutant were co-cultured with Caco-2 intestinal epithelial cells (IECs) in a vertical diffusion chamber (VDC) at an MOI of 100:1 for 24 h with either aerobic (white bars) or microaerobic (black bars) conditions in the apical compartment. The numbers of interacting bacteria were determined by lysis of the IECs after co-culturing, serial dilution and plating of the lysates (A). The numbers of intracellular bacteria were determined by killing of any extracellular bacteria with gentamicin followed by lysis of the IECs and serial dilution and plating of the lysates (B).

The numbers of interacting and intracellular 11168H *ciaB* mutant bacteria did not differ from those observed for the *C. jejuni* 11168H wild-type strain after 24 h of co-culturing in the VDC with either aerobic or microaerobic conditions in the apical compartment. This suggests that either CiaB is not involved in mediating the increased interaction and invasion observed in the VDC model or that other virulence factors compensate for the *ciaB* mutation over the relatively long co-incubation period of 24 h, especially as the original report demonstrating the involvement of CiaB in IEC invasion by *C. jejuni* utilised shorter co-culturing times (30 mins for interaction and 3 h for invasion (Konkel *et al.*, 1999a). As the original report also states that a *C. jejuni* F38011 *ciaB* mutant is defective in secretion of all Cia proteins (Konkel *et al.*, 1999b), the results shown here would suggest that the Cia proteins as such are not involved in the increased interaction and invasion of IECs by *C. jejuni* co-cultured with microaerobic conditions in the apical compartment of the VDC. This data is in contrast to the original reports on CiaB and also to recent data obtained with this *C. jejuni* 11168H *ciaB* mutant at the LSHTM (Nevada Naz, personal communication), but in accordance with the recent analysis of an 81-176 *ciaB* mutant (Novik *et al.*, 2010). This merits further investigation into the numbers of interacting and intracellular bacteria for the 11168H *ciaB* mutant and a 81-176 *ciaB* mutant in the VDC model, especially at shorter co-culturing time points. However, due to time and throughput constraints, this was not pursued further in this study.

There are two possible explanations for the data presented above. One is that CiaB has no role in *C. jejuni* interactions with and invasion of IECs and therefore the finding that a 11168H *ciaB* mutant does not exhibit lower numbers of interacting and intracellular bacteria in the VDC model compared to the *C. jejuni* 11168H wild-type strain is an accurate observation. Alternatively it is possible that the VDC model does not allow the detection of differences between numbers of interacting and intracellular bacteria between a *C. jejuni* strain and a mutant after 24 h of co-culturing. To confirm that the VDC model was able to detect differences in the invasive phenotype of a *C. jejuni* mutant, 11168H *flaA* and *rpoN* mutants were analysed in the VDC model.

6.3 Two non-motile *C. jejuni* 11168H mutants exhibit reduced numbers of interacting and intracellular bacteria when co-cultured with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment

As stated above, the role of CiaB is not without controversy within the *Campylobacter* research community. On the other hand, motility has been widely reported to be essential for *C. jejuni* to interact with and especially to invade IECs. To identify whether motility under microaerobic conditions was important, the interaction and invasion numbers of two non-motile mutants were analysed. The two mutants chosen were the *C. jejuni* 11168H *flaA* and *rpoN* mutants.

6.3.1 A 11168H *flaA* mutant exhibits reduced numbers of interacting and intracellular bacteria when co-cultured with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment

The *C. jejuni flaA* gene encodes the major flagellin subunit protein FlaA. Together with FlaB, the FlaA protein makes up the flagellar filament (Guerry *et al.*, 1991). Regulation of *C. jejuni* flagellar biogenesis is highly complex and is controlled in part by the alternative sigma factors σ^{54} (RpoN) and σ^{28} (FliA). Additionally, the anti-sigma factor-like protein FlgM has been demonstrated to be a temperature-dependent regulator of both σ^{54} - and σ^{28} - dependent flagellin expression (Wosten *et al.*, 2010b). A *C. jejuni* F38011 *flaA* mutant has been demonstrated to be non-motile and unable to secrete the Cia proteins (Konkel *et al.*, 2004). A *C. jejuni* 81116 *flaA* mutant has previously been demonstrated to exhibit a reduction in host cell invasion (Wassenaar *et al.*, 1991), and the role of the major flagellin subunit protein FlaA in *C. jejuni* IEC invasion is widely accepted. As such, a 11168H *flaA* mutant can serve as a robust control.

A 11168H *flaA* mutant was obtained from the LSHTM *Campylobacter jejuni* mutant library (<http://crf.lshtm.ac.uk/>) and insertion of the Kan^R cassette into the *flaA* gene confirmed by PCR (Figure 6.3).

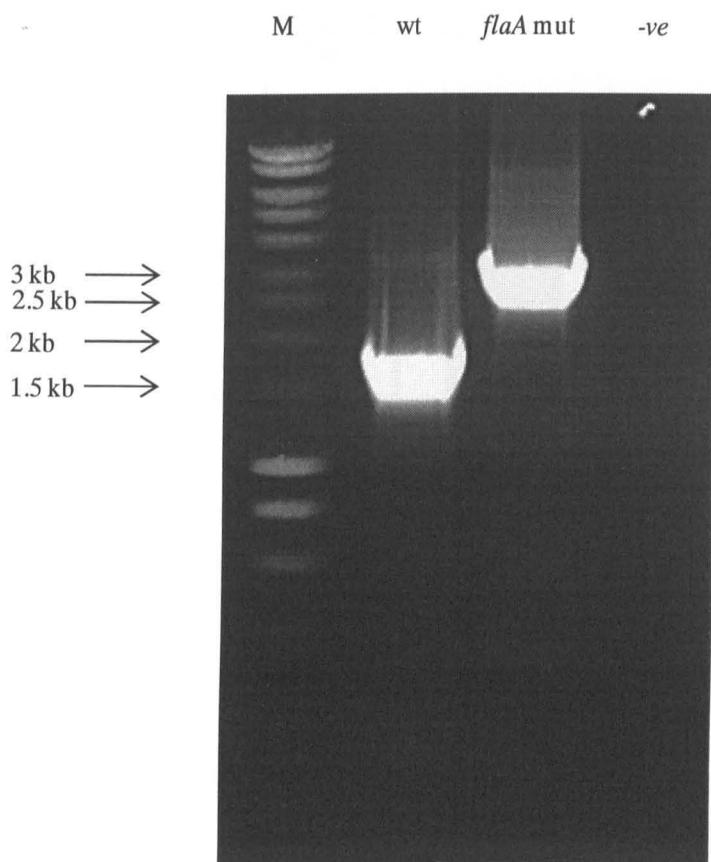
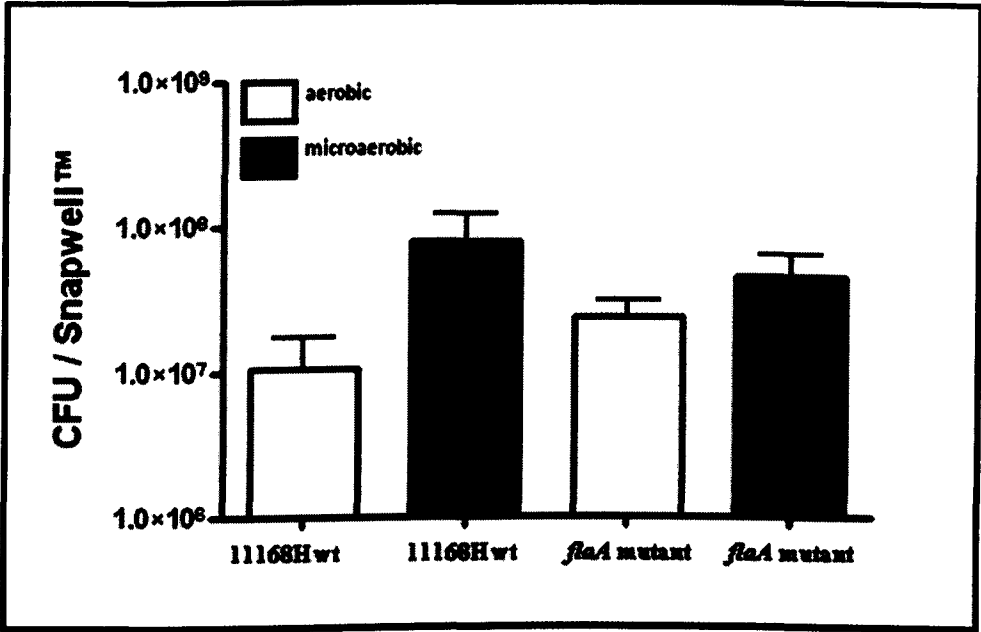


Figure 6.3: Genomic DNA was isolated from the *C. jejuni* 11168H wild-type strain and the 11168H *flaA* mutant. Primers annealing to the 5'- and the 3'-end of the *flaA* gene were used to amplify PCR products of 1.5 kb and 2.9 kb from wild-type strain and mutant genomic DNA templates, respectively, demonstrating successful disruption of the *flaA* gene in the 11168H *flaA* mutant.

After verifying mutation of the *flaA* gene, the 11168H *flaA* mutant was co-cultured with Caco-2 IECs in the VDC for 24 h with either aerobic or microaerobic conditions in the apical compartment and the numbers of interacting bacteria determined by lysis of the IECs post incubation and serial dilution and plating of the lysates (Figure 6.4A). The number of intracellular bacteria was determined by killing the extracellular bacteria with gentamicin prior to IEC lysis as described in section 2.10.4 (Figure 6.4B). The numbers obtained were compared to those previously obtained with the *C. jejuni* 11168H wild-type strain (See sections 5.2 and 5.3).

A



B

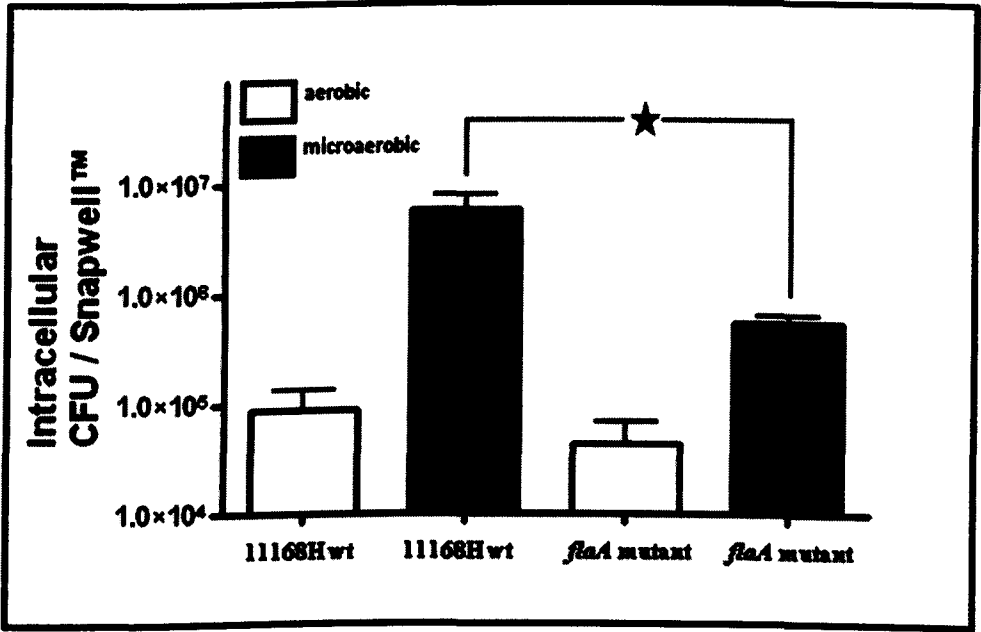


Figure 6.4: *C. jejuni* 11168H wild-type strain and 11168H *flaA* mutant bacteria were co-cultured with Caco-2 intestinal epithelial cells (IECs) in a vertical diffusion chamber (VDC) at an MOI of 100:1 for 24 h with either aerobic (white bars) or microaerobic (black bars) conditions in the apical compartment. The numbers of interacting bacteria were determined by lysis of the IECs after co-culturing, serial dilution and plating of the lysates (A). The numbers of intracellular bacteria were determined by killing of any extracellular bacteria with gentamicin followed by lysis of the IECs and serial dilution and plating of the lysates (B). * = $p < 0.05$

The numbers of interacting 11168H *flaA* mutant bacteria were slightly higher than those observed for *C. jejuni* 11168H wild-type strain when co-cultured with the Caco-2 IECs in the VDC with aerobic conditions in the apical compartment (Figure 6.4A). However, when co-cultured with microaerobic conditions in the apical compartment, the numbers of interacting *flaA* mutant bacteria were significantly lower than those observed for the *C. jejuni* 11168H wild-type strain. Furthermore, under microaerobic conditions, the 11168H *flaA* mutant demonstrated a reduced increase in interacting bacteria compared to the wild-type strain (Figure 6.4A, compare differences between columns 1-2 and 3-4), suggesting the 11168H *flaA* mutant was no longer able to respond to the microaerobic conditions in the same way as the wild-type strain. The numbers of intracellular 11168H *flaA* mutant bacteria were lower than those observed for the *C. jejuni* 11168H wild-type when co-cultured with Caco-2 IECs in the VDC independent of the conditions in the apical compartment, with the difference reaching statistical significance after co-culturing with microaerobic conditions in the apical compartment (Figure 6.4B). Furthermore, the 11168H *flaA* mutant demonstrated a reduced increase in the numbers of intracellular bacteria when co-cultured with microaerobic conditions in the apical compartment compared to the wild-type strain (Figure 6.4B, compare differences between columns 1-2 and 2-3), suggesting the 11168H *flaA* mutant was no longer able to respond to the microaerobic conditions in the same way as the *C. jejuni* 11168H wild-type strain in terms of IEC invasion.

These differences suggest that bacterial motility during co-culturing with IECs in the VDC with microaerobic conditions in the apical compartment is an important determinant of the increased interaction and invasion of the *C. jejuni* 11168H wild-type strain compared to aerobic conditions.

6.3.2 A 11168H *rpoN* mutant exhibits reduced numbers of interacting and intracellular bacteria when co-cultured with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment

Flagellar gene transcription in *C. jejuni* has been demonstrated to be regulated in part by the two alternate sigma factors, σ^{28} and σ^{54} . While expression of the major flagellar subunit FlaA is regulated by σ^{28} (FlaA), the expression of the minor flagellar

subunit FlaB as well as the flagellar hook protein FlgE are regulated by σ^{54} (RpoN) (Jagannathan *et al.*, 2001, Kamal *et al.*, 2007). A 11168H *rpoN* mutant has been demonstrated to be aflagellate and non-motile (Fernando *et al.*, 2007, Jagannathan *et al.*, 2001). Further studies of a *C. jejuni* 11168H *rpoN* exhibited a reduction in HeLa cell interaction and invasion under standard tissue culture conditions, as well as reduced CFUs in chick colonisation experiments (Fernando *et al.*, 2007). Microarray analysis of the gene expression profile of the *C. jejuni* 11168H *rpoN* mutant identified multiple differentially expressed genes (both flagellar and non-flagellar related) compared to the wild-type strain (Kamal *et al.*, 2007).

A 11168H *rpoN* mutant was obtained from Dr Nick Dorrell (LSHTM) and screened for insertion of the Kan^R cassette into the *rpoN* gene by PCR (Figure 6.5). Approximately 900 bp of the *rpoN* gene had been deleted in the construction of the mutant, so the difference in PCR product size between the wild-type and the mutant was expected to be approximately 500 bp (Nick Dorrell, personnel communication).

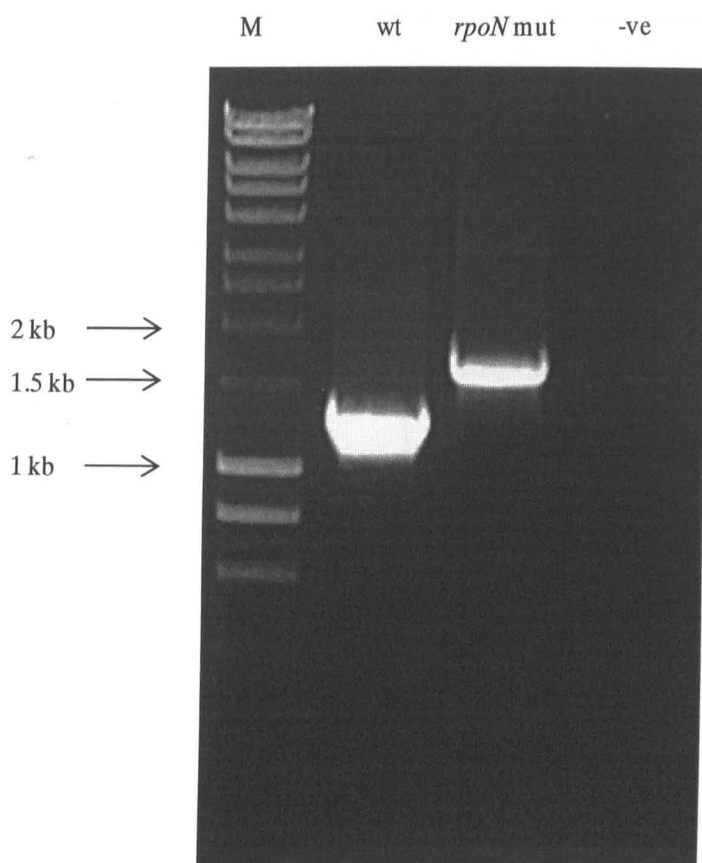
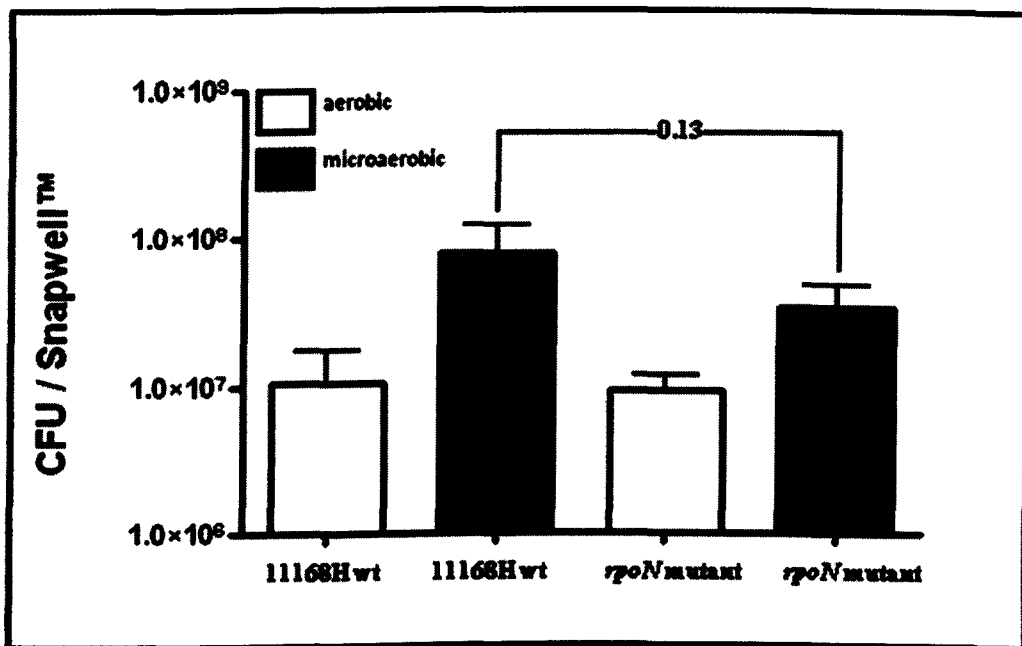


Figure 6.5: Genomic DNA was isolated from the *C. jejuni* 11168H wild-type strain and a 11168H *rpoN* mutant strain. As 900 bp of the *rpoN* gene had been deleted during construction of the mutant, a difference in PCR product size of 500 bp was expected for the insertional mutant. Primers annealing to the 5'- and the 3'-end of the *rpoN* gene were used to amplify PCR products of 1.2 kb and 1.7 kb from the wild-type strain and the mutant genomic DNA templates, respectively, demonstrating successful disruption of the *rpoN* gene in the 11168H *rpoN* mutant.

After verifying mutation of the *rpoN* gene, the 11168H *rpoN* mutant was co-cultured with Caco-2 IECs in the VDC for 24 h with either aerobic or microaerobic conditions in the apical compartment, and the numbers of interacting bacteria determined by lysis of the IECs post incubation and serial dilution and plating of the lysates (Figure 6.6A). The number of intracellular bacteria was determined by killing the extracellular bacteria with gentamicin prior to IEC lysis as described in section 2.10.4 (Figure 6.6B). The numbers obtained were compared to those previously obtained with the *C. jejuni* 11168H wild-type strain (See sections 5.2 and 5.3).

A



B

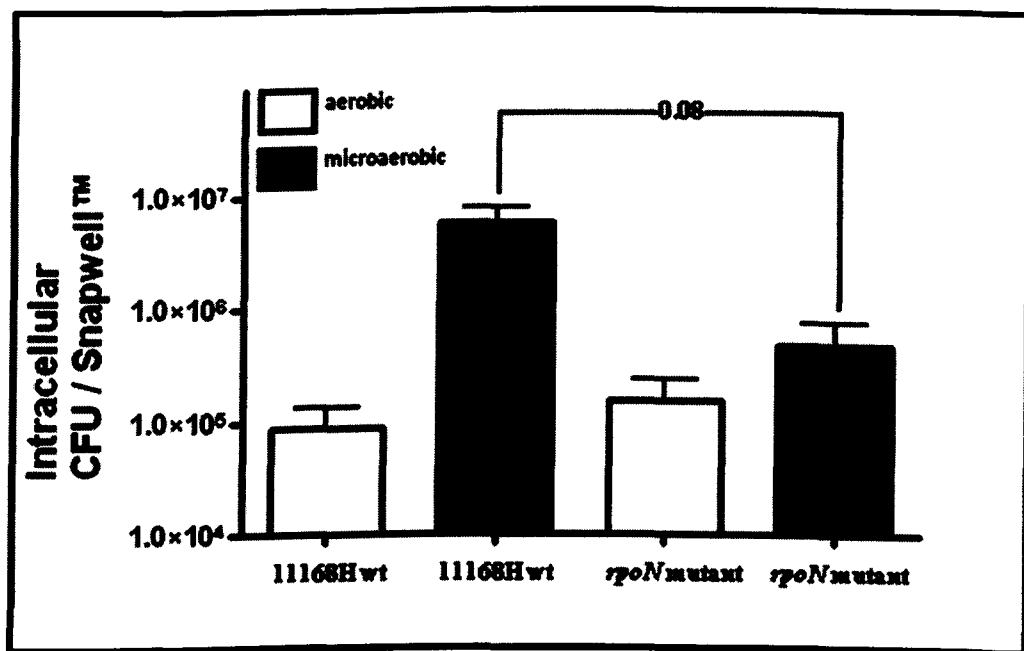


Figure 6.6: *C. jejuni* 11168H wild-type strain and 11168H *rpoN* mutant bacteria were co-cultured with Caco-2 intestinal epithelial cells (IECs) in a vertical diffusion chamber (VDC) at an MOI of 100:1 for 24 h with either aerobic (white bars) or microaerobic (black bars) conditions in the apical compartment. The numbers of interacting bacteria were determined by lysis of the IECs after co-culturing, serial dilution and plating of the lysates (A). The numbers of intracellular bacteria were determined at both time points by killing of any extracellular bacteria with gentamicin followed by lysis of the IECs and serial dilution and plating of the lysates (B).

The numbers of interacting 11168H *rpoN* mutant bacteria did not differ from the numbers observed for the *C. jejuni* 11168H wild-type strain when co-cultured with Caco-2 IECs in the VDC for 24 h with aerobic conditions in the apical compartment (Figure 6.6A, compare columns 1 with 3). However, when co-cultured with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment, the numbers of interacting 11168H *rpoN* mutant bacteria were lower than those obtained for the *C. jejuni* 11168H wild-type bacteria (Figure 6.6A, compare columns 2 with 4). Furthermore and similar to the *flaA* mutant, under microaerobic conditions the 11168H *rpoN* mutant demonstrated a reduced increase in interacting bacteria compared to the wild-type strain (Figure 6.6A, compare differences between columns 1-2 and 3-4), suggesting the 11168H *rpoN* mutant was no longer able to respond to the microaerobic conditions in the same way as the *C. jejuni* 11168H wild-type strain

The numbers of intracellular 11168H *rpoN* mutant bacteria when co-cultured with Caco-2 IECs with aerobic conditions in the apical compartment of the VDC were slightly higher than those obtained with the *C. jejuni* 11168H wild-type strain, however the difference was not statistically significant (Figure 6.6B, compare columns 1 with 3). When co-cultured with microaerobic conditions in the apical compartment, however, the numbers of intracellular 11168H *rpoN* mutant bacteria were approximately ten times lower than those obtained for the *C. jejuni* 11168H wild-type strain (Figure 6.6B, compare columns 2 with 4). Furthermore under microaerobic conditions, the 11168H *rpoN* mutant demonstrated a reduced increase in the numbers of intracellular bacteria compared to the wild-type strain (Figure 6.6B, compare differences between columns 1-2 and 3-4) suggesting the 11168H *rpoN* mutant was no longer able to respond to the microaerobic conditions in terms of invasion in the same way as the *C. jejuni* 11168H wild-type strain.

Taken together, the findings presented above demonstrate that under these experimental conditions and at 24 h post-infection, the 11168H *flaA* and *rpoN* mutants exhibited lower numbers of interacting and intracellular bacteria after 24 h of co-culturing in the VDC compared to the *C. jejuni* 11168H wild-type strain, in particular with microaerobic conditions in the apical compartment. Intriguingly, both the *flaA* and *rpoN* mutants demonstrated a reduced increase in the numbers of intracellular bacteria under microaerobic conditions compared to the wild-type strain, indicating that motility is one of the factors leading to increased interaction

with and invasion of the IECs observed for the *C. jejuni* 11168H wild-type strain when co-cultured with microaerobic conditions at the apical surface.

6.4 The phenotypes of the two non-motile *C. jejuni* 11168H mutants with respect to interaction and invasion of IECs after co-culturing with Caco-2 IECs in the VDC are more pronounced after a shorter period of co-culturing

All experiments described above utilised the relatively long co-incubation time of 24 h. This time point was initially chosen as the differences between the numbers of interacting and intracellular *C. jejuni* 11168H wild-type strain bacteria recovered after co-culturing with IECs with microaerobic conditions at the apical surface were the most significant (See Chapter 5). However, most common *in vitro* co-culturing models utilise far shorter co-incubation times. Therefore, the numbers of interacting and intracellular bacteria were assessed at 6 h co-incubation for the 11168H *flaA* and the *rpoN* mutants.

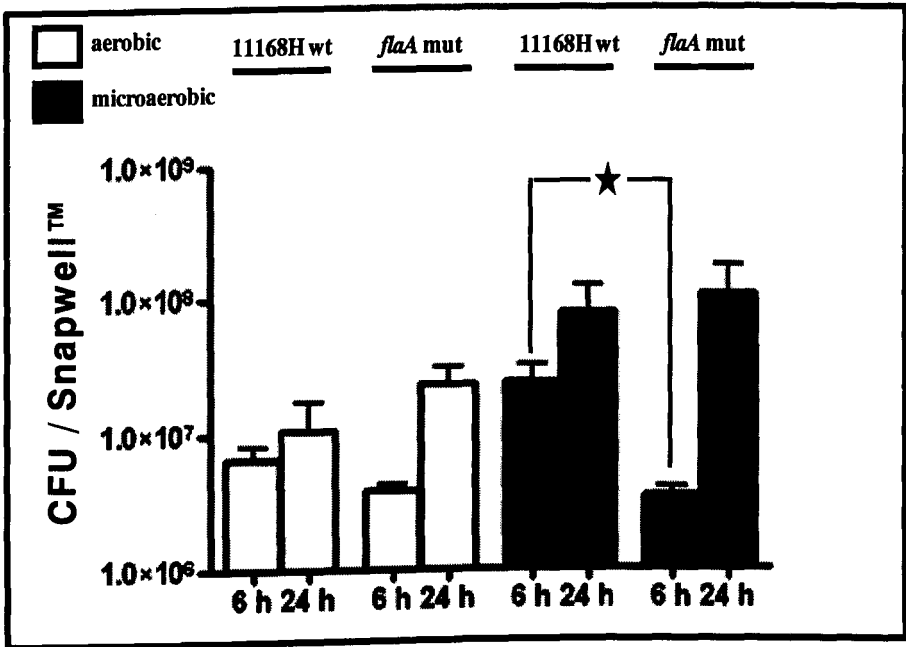
6.4.1 The phenotype of the 11168H *flaA* mutant with respect to interaction and invasion of IECs after co-culturing with Caco-2 IECs in the VDC is more pronounced after a shorter period of co-culturing

The 11168H *flaA* mutant was co-cultured with Caco-2 IECs in the VDC with either aerobic or microaerobic conditions in the apical compartment for 6 h and the numbers of interacting and intracellular bacteria determined.

After 6 h of co-culturing, the numbers of interacting 11168H *flaA* mutant bacteria when co-cultured with Caco-2 IECs in the VDC were the same independent of whether the bacteria had been had been co-cultured with aerobic or microaerobic conditions in the apical compartment of the VDC (Figure 6.7A, compare columns 3 and 7). Furthermore, the numbers were lower than those observed for the *C. jejuni* 11168H wild-type strain (Figure 6.7A, compare columns 1 with 3 for aerobic and 5 with 7 for microaerobic). Intriguingly, when compared with the data obtained after

24 h of co-culturing, the 11168H *flaA* demonstrated a greater increase in the number of interacting bacteria between time points 6 h and 24 h compared to the *C. jejuni* 11168H wild-type strain, independent of the co-culturing condition (Figure 6.7A, compare all column pairs). Furthermore, the increase in bacterial numbers between the 6 h and the 24 h time points was more pronounced after co-culturing with microaerobic conditions at the apical surface. This suggests that i: aerobic co-culturing may “mask” a phenotype and ii: in the case of the 11168H *flaA* mutant, longer co-culturing allows for compensation of the “lower interacting” phenotype.

A



B

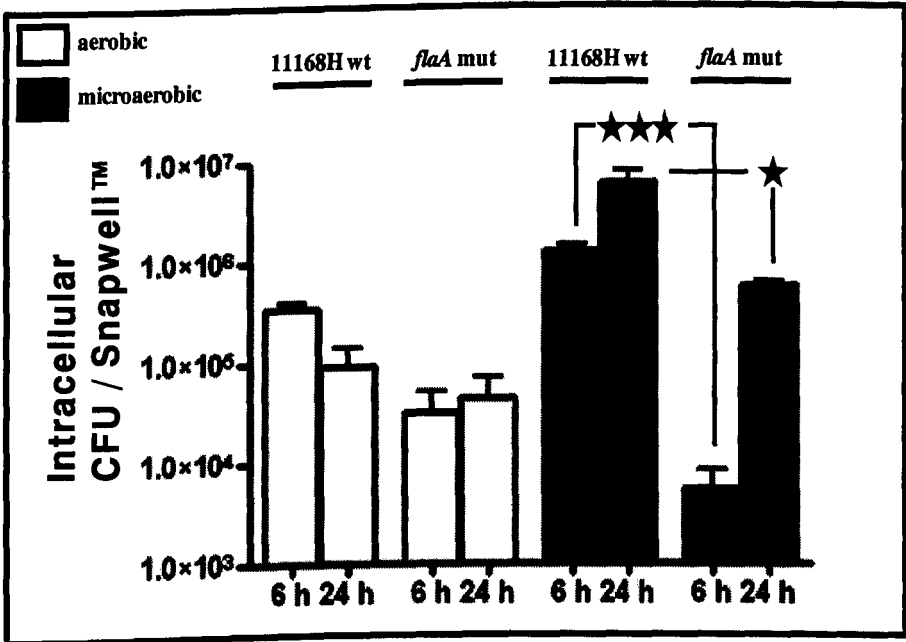


Figure 6.7: *C. jejuni* 11168H wild-type strain and 11168H *flaA* mutant bacteria were co-cultured with Caco-2 intestinal epithelial cells (IECs) in a vertical diffusion chamber (VDC) at an MOI of 100:1 for 24 h or 6 h with either aerobic (white bars) or microaerobic (black bars) conditions in the apical compartment. The numbers of interacting bacteria were determined by lysis of the IECs after co-culturing, serial dilution and plating of the lysates (A). The numbers of intracellular bacteria were determined at both time points by killing of any extracellular bacteria with gentamicin followed by lysis of the IECs and serial dilution and plating of the lysates (B). * = $p < 0.05$, *** = $p < 0.001$

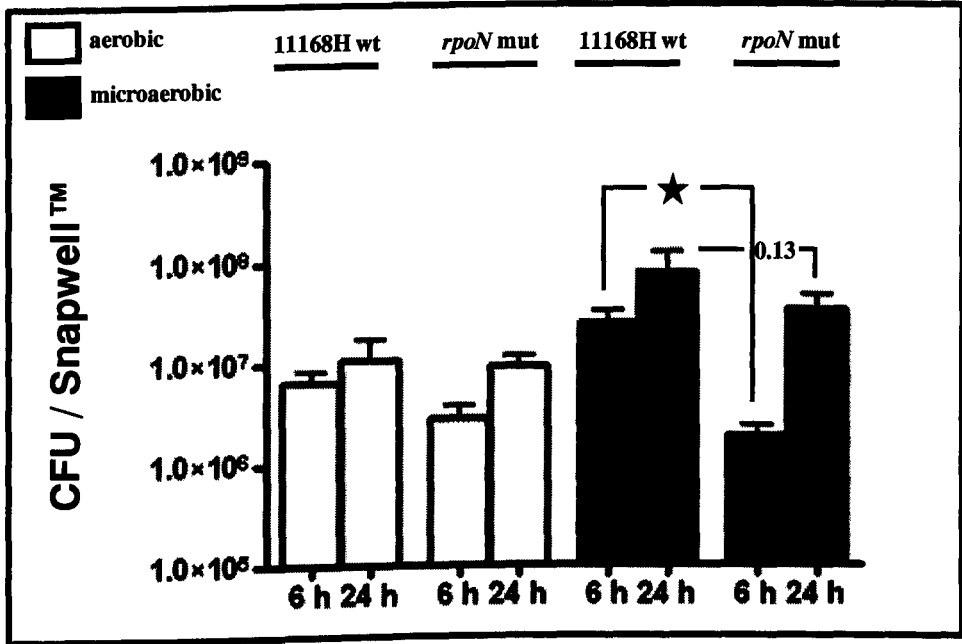
Previous published data demonstrated that a *C. jejuni* 81116 *flaA* mutant exhibited markedly reduced potential for IEC invasion (Wassenaar *et al.*, 1991). After 24 h of co-culturing with Caco-2 IECs in the VDC, the number of intracellular 11168H *flaA* mutant bacteria was lower than the number obtained for *C. jejuni* 11168H wild-type strain, independent of the co-culturing conditions (Figure 6.7B, compare columns 2 with 4 and 6 with 8). After 6 h of co-culturing, the difference between the 11168H *flaA* mutant and the wild-type strain was more pronounced (Figure 6.7B, compare columns 1 with 3 and columns 5 with 7). Intriguingly, after 6 h of co-culturing, the numbers of intracellular 11168H *flaA* mutant bacteria were higher for experiments performed with aerobic conditions in the apical compartment of the VDC compared to the experiments performed with microaerobic conditions in the apical compartment (Figure 6.7B, compare columns 3 with 7 for the mutant and columns 1 with 5 for the wild-type strain), an inversion of all previously obtained data. Additionally, when compared with the data obtained after 24 h of co-culturing, the 11168H *flaA* mutant appeared to have partially recovered under microaerobic conditions but not under aerobic conditions (Figure 6.7B, compare differences between columns 3-4 for aerobic and 7-8 for microaerobic).

This data suggests that bacterial motility is not only important for IEC invasion by *C. jejuni* per se, but that the observed increased invasion after co-culturing *C. jejuni* 11168H wild-type strain with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment compared to aerobic conditions may also be linked to the motility of the bacteria. Hence, a non-motile bacterial mutant loses the ability to respond to the microaerobic, *in vivo*-like conditions and is less invasive when co-cultured with IECs under microaerobic conditions. Interestingly, with regards to both interacting and intracellular bacteria, the phenotype of the 11168H *flaA* mutant is more pronounced at shorter co-culturing time points, suggesting that motility is particularly important at early phases of interaction and invasion, and that longer co-incubations allow for partial compensation of the phenotype, possibly through the action of other bacterial factors.

6.4.2 The phenotype of the 11168H *rpoN* mutant with respect to interaction and invasion of IECs after co-culturing with Caco-2 IECs in the VDC is more pronounced after a shorter period of co-culturing

After 24 h of co-incubation, lower numbers of interacting and intracellular bacteria were observed for the 11168H *rpoN* mutant compared to the *C. jejuni* 11168H wild-type strain (Figure 6.4). Furthermore, the 11168H *rpoN* mutant demonstrated a reduced increase in the number of interacting and intracellular bacteria after co-culturing with Caco-2 IECs in the VDC under microaerobic conditions compared to the wild-type strain. To analyse the phenotype of the 11168H *rpoN* mutant after a shorter co-culturing time, the 11168H *rpoN* mutant was co-cultured with Caco-2 IECs in the VDC for 6 h with either aerobic or microaerobic conditions in the apical compartment and the numbers of interacting and intracellular bacteria determined

A



B

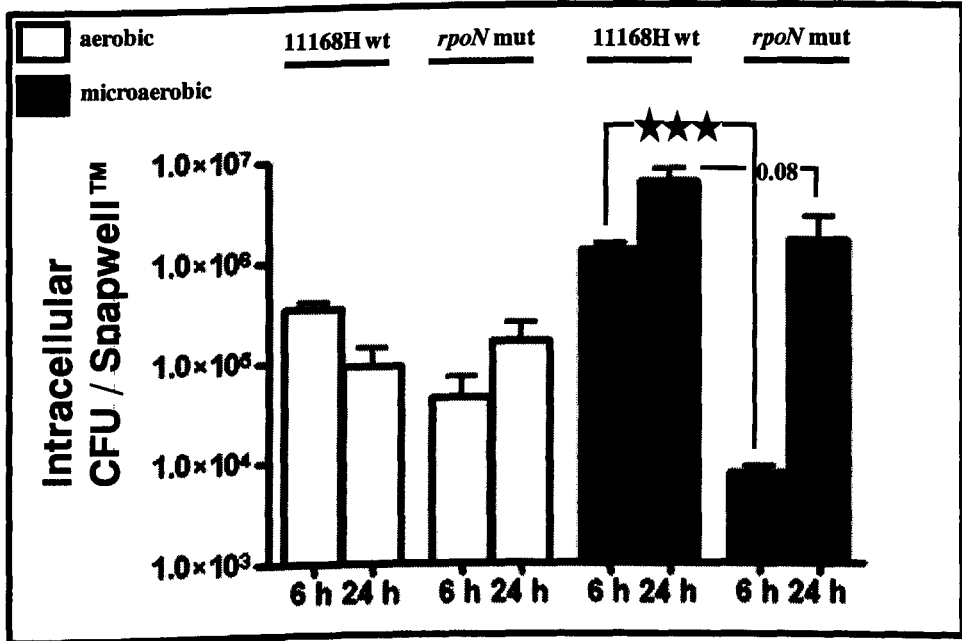


Figure 6.8: *C. jejuni* 11168H wild-type strain and 11168H *rpoN* mutant bacteria were co-cultured with Caco-2 intestinal epithelial cells (IECs) in a vertical diffusion chamber (VDC) at an MOI of 100:1 for 6 h with either aerobic (white bars) or microaerobic (black bars) conditions in the apical compartment. The numbers of interacting bacteria were determined by lysis of the IECs after co-culturing, serial dilution and plating of the lysates (A). The numbers of intracellular bacteria were determined at both time points by killing of any extracellular bacteria with gentamicin followed by lysis of the IECs and serial dilution and plating of the lysates (B). * = $p < 0.05$, *** = $p < 0.001$

After 6 h of co-culture, the numbers of interacting 11168H *rpoN* mutant bacteria were lower than those obtained for *C. jejuni* 11168H wild-type strain, independent of the co-culturing conditions (Figure 6.8A, compare columns 1 with 3 and columns 4 with 7). Intriguingly, the numbers of interacting 11168H *rpoN* mutant bacteria were lower after co-culturing with microaerobic compared to aerobic conditions in the apical compartment of the VDC (Figure 6.8A, compare columns 3 with 7). This differed from the results obtained for the non-motile 11168H *flaA* mutant, where the numbers of interacting bacteria were approximately the same after 6 h of co-culturing in both conditions (Figure 6.7A, compare columns 3 with 7).

The numbers of intracellular 11168H *rpoN* mutant bacteria were also assessed after 6 h of co-culturing. The number of intracellular 11168H *rpoN* mutant bacteria was lower than the number obtained for the *C. jejuni* 11168H wild-type strain, independent of the co-culturing conditions (Figure 6.8B, compare columns 1 with 3 and columns 4 with 7). Furthermore, in contrast to the *C. jejuni* 11168H wild-type strain, the number of intracellular 11168H *rpoN* mutant bacteria was lower after co-culturing with microaerobic conditions in the apical compartment of the VDC compared to co-culturing with aerobic condition in the apical compartment (Figure 6.8B, compare columns 1 with 5 and columns 3 with 7), which again represents an inversion of the data obtained with the *C. jejuni* 11168H wild-type strain.

In summary, lower numbers of interacting and intracellular 11168H *flaA* and *rpoN* mutant bacteria were observed after 6 h of co-culturing with Caco-2 IECs in the VDC compared to the numbers obtained with the *C. jejuni* 11168H wild-type strain, independent of the co-culturing condition. However, regarding the differences between the aerobic and the microaerobic co-culturing conditions, roughly equal numbers of interacting 11168H *flaA* mutant bacteria were observed under both conditions, in contrast to the wild-type strain, where higher numbers of interacting bacteria were recovered under after co-culturing with microaerobic conditions in the apical compartment. Furthermore, lower numbers of intracellular 11168H *flaA* mutant bacteria were observed after co-culturing under microaerobic compared to aerobic conditions. This is the opposite of the wild-type strain, where higher numbers of intracellular bacteria were found after co-culturing under microaerobic compared to aerobic conditions. This phenotype was more pronounced for the 11168H *rpoN* mutant, where lower numbers of interacting as well as lower numbers

of intracellular bacteria were found after co-culturing under microaerobic compared to aerobic conditions.

These findings suggest that motility is essential, but not the only factor involved in the increased bacterial invasion under microaerobic conditions in the VDC. Even though both mutants are non-motile, there are a number of phenotypic differences due to the two mutations. Whilst the *flaA* mutant lacks the major flagellin subunit protein FlaA, a F38001 *flaA* mutant has been shown to still possess a truncated flagellar filament and to be secretion positive (Konkel *et al.*, 2004). On the other hand, the *rpoN* mutant has been shown to be completely aflagellate, microarray analysis revealed multiple transcriptional changes, including genes not involved in flagellar biosynthesis and the mutant has been shown to be secretion negative (Kamal *et al.*, 2007, Fernando *et al.*, 2007).

As *rpoN* codes for one of the two alternative sigma factors of *C. jejuni*, it is possible that the *rpoN* mutant is unable to modulate the transcription of a plethora of other genes under the control of RpoN. If the regulation of any of these genes is changed during co-culturing with IECs in the VDC with microaerobic conditions in the apical compartment in the *C. jejuni* 11168H wild-type strain resulting in increased interaction and invasion of the IECs by bacteria, it can be hypothesised that the 11168H *rpoN* mutant, which is unable to modulate the expression of these genes due to the absence of σ^{54} , would no longer be able to exhibit an increased interaction and invasion.

6.5 Complementation of the *rpoN* mutation gene partially restores the phenotype of the 11168H *rpoN* mutant

As *C. jejuni* does not readily support episomal elements such as plasmid vectors, complementation requires the insertion of a functional copy of the gene back into the chromosome. A vector described recently, pCAM148, (Hitchen *et al.*, 2010) was used to complement the *rpoN* mutation in the 11168H *rpoN* mutant. The vector was engineered to carry a 2179 bp fragment corresponding to nucleotides 205297–207475 of the *C. jejuni* NCTC11168 genome. This stretch of nucleotides was originally annotated as a probable pseudogene (*Cj0223*), and this was confirmed by the re-annotation of the *C. jejuni* NCTC11168 genome (Gundogdu *et al.*, 2007). Within the 2179 bp fragment, the vector was engineered to carry a chloramphenicol

resistance cassette lacking a transcriptional terminator, followed by a small multiple cloning site (MCS) comprised of recognition sites for the restriction endonucleases *NcoI* and *NheI*. The gene to be complemented can be directionally cloned into the MCS and will be expressed from the constitutively active Cam^R promoter.

6.5.1 Cloning of the *rpoN* gene into the complementation vector

pCAM148

The *rpoN* gene was amplified by PCR from *C. jejuni* 11168H wild-type strain genomic DNA using a forward primer that had been designed to anneal 15 bases upstream of the ATG start codon in order to include the ribosomal binding site and a reverse primer that annealed to the 3' end of the *rpoN* gene.

The PCR product was purified, digested with *NcoI* and *NheI* and ligated between the *NcoI/NheI* sites of the complementation vector. Successful cloning was confirmed by purifying plasmid pCAM148::*rpoN* from *E. coli* and digesting the purified plasmid with *NcoI* and *NheI*. Release of a DNA fragment of the correct size from both plasmids, but not from the original empty vector used for cloning, demonstrated the successful cloning of a fragment of the correct size into the complementation vector (Figure 6.9). Sequencing of the insert confirmed successful cloning of the *C. jejuni* 11168H *rpoN* gene into pCAM148 (data not shown).

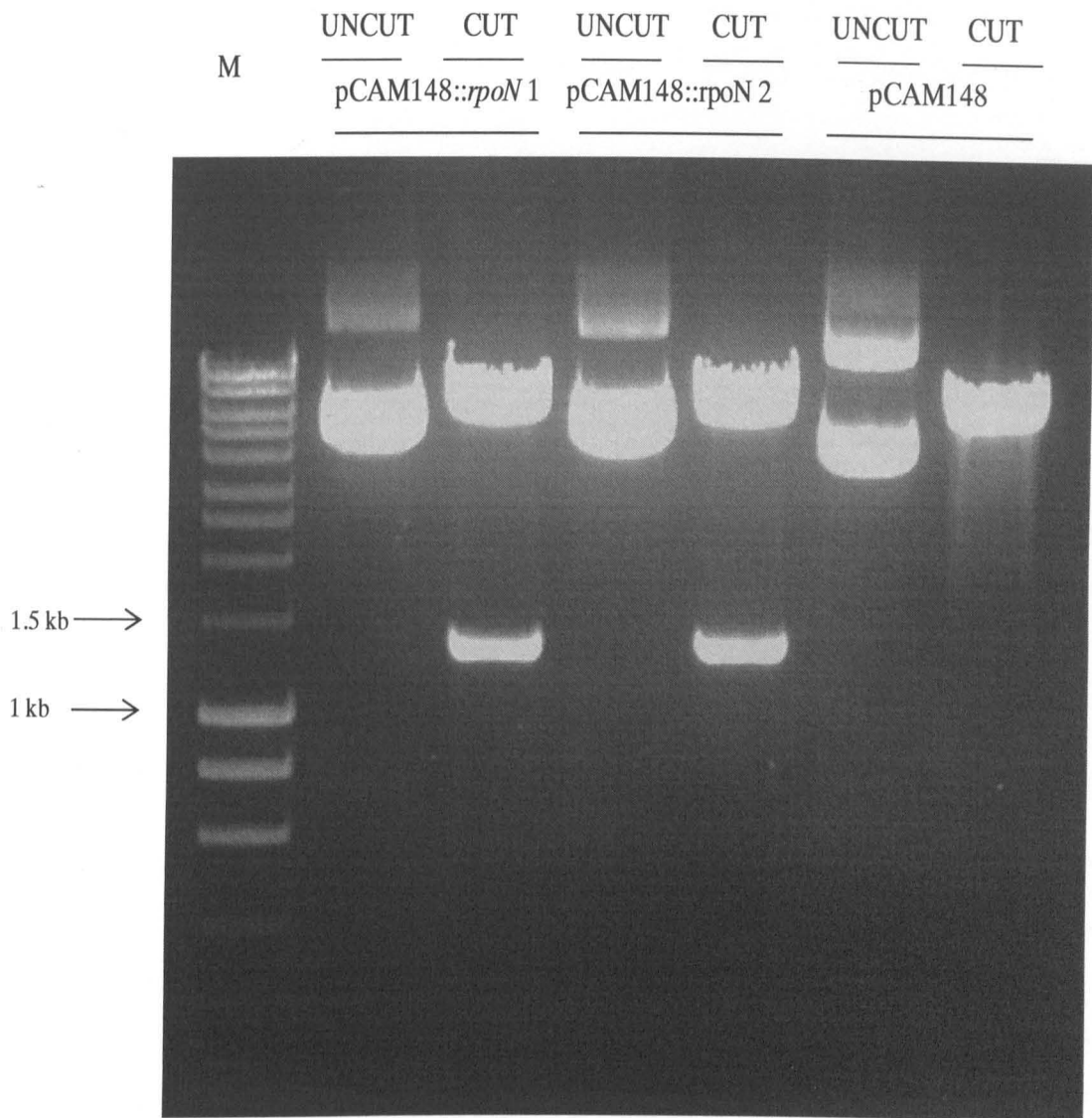


Figure 6.9: Two clones of plasmid DNA of 11168H *rpoN* cloned into pCAM148 as well as the empty pCAM148 vector were digested with *NcoI* and *NheI* and the fragments separated by agarose gel electrophoresis (CUT). Uncut versions of each plasmid were included as controls (UNCUT). Release of a fragment of 1.3 kb from both pCAM148 constructs containing the *rpoN* insert confirmed the cloning of a DNA fragment of the correct size into pCAM148.

6.5.2 Transformation of the 11168H *rpoN* mutant with the complementation construct

Clone 1 of the complementation plasmids was introduced into the 11168H *rpoN* mutant by electroporation as described in section 2.9.10. Chloramphenicol resistant clones were analysed by PCR using the primers used to originally amplify the *rpoN*

gene from *C. jejuni* 11168H genomic DNA. The presence of both a wild-type and a mutant PCR product in both clones analysed confirmed the presence of the complementation construct (Figure 6.10).

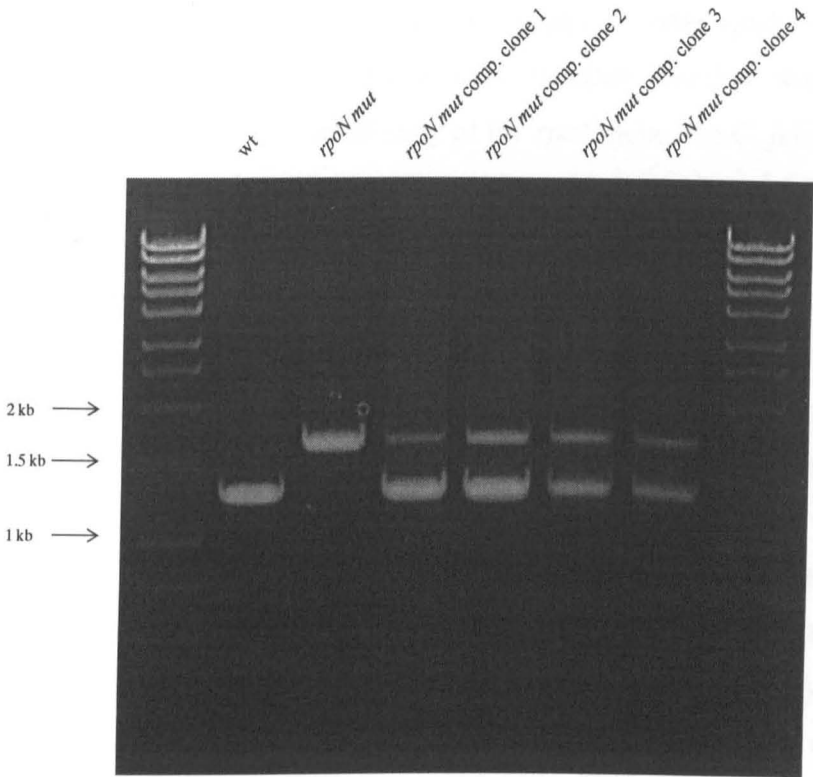


Figure 6.10: Chromosomal DNA was prepared from the *C. jejuni* 11168H wild-type strain, the 11168H *rpoN* mutant and four clones of 11168H *rpoN* mutant transformed with the complementation construct. PCR reactions were performed using primers annealing to the 5' and the 3' end of the *rpoN* gene sequence and the products analysed by agarose gel electrophoresis. The presence of both the wild-type and the mutant PCR products in all four complemented clones demonstrated the successful reintroduction of the *rpoN* gene back into the 11168H *rpoN* mutant.

Prior to use of the complemented 11168H *rpoN* mutant in VDC assays, two other experiments were performed to confirm that the mutation was not only genetically but also functionally complemented.

6.5.3 The motility phenotype of the 11168H *rpoN* mutant is partially complemented by expression of a functional copy of *rpoN*

Motility is a phenotype that can be assessed using soft agar swarming plates. A previous study has shown that a *C. jejuni* 11168H *rpoN* mutant is non-motile (Fernando *et al.*, 2007). To analyse whether motility was complemented by reintroduction of a functional copy of the *rpoN* gene, the *C. jejuni* 11168H wild-type strain, the 11168H *rpoN* mutant as well as the complemented 11168H *rpoN* mutant were assessed for motility as described in section 2.11.1. As expected, the *C. jejuni* 11168H wild-type strain displayed normal motility, while the 11168H *rpoN* mutant was non-motile. The complemented 11168H *rpoN* mutant showed some motility, albeit less than the wild-type, suggesting that only partial complementation was occurring (Figure 6.11). This finding was not unexpected, as the *rpoN* mutation will most likely effect the expression of several other genes, which may not be completely rescued by the recombinant protein. Furthermore, it has been demonstrated that the FlgRS two-component regulatory system is the master regulator of the *Campylobacter jejuni* flagellum hierarchy and involved in controlling the expression of *rpoN* (Wosten *et al.*, 2004). In the complemented *rpoN* mutant, however, *rpoN* is under the control of a constitutively active promoter, which may have an influence on the phenotype due to different effects on genes regulated by RpoN.

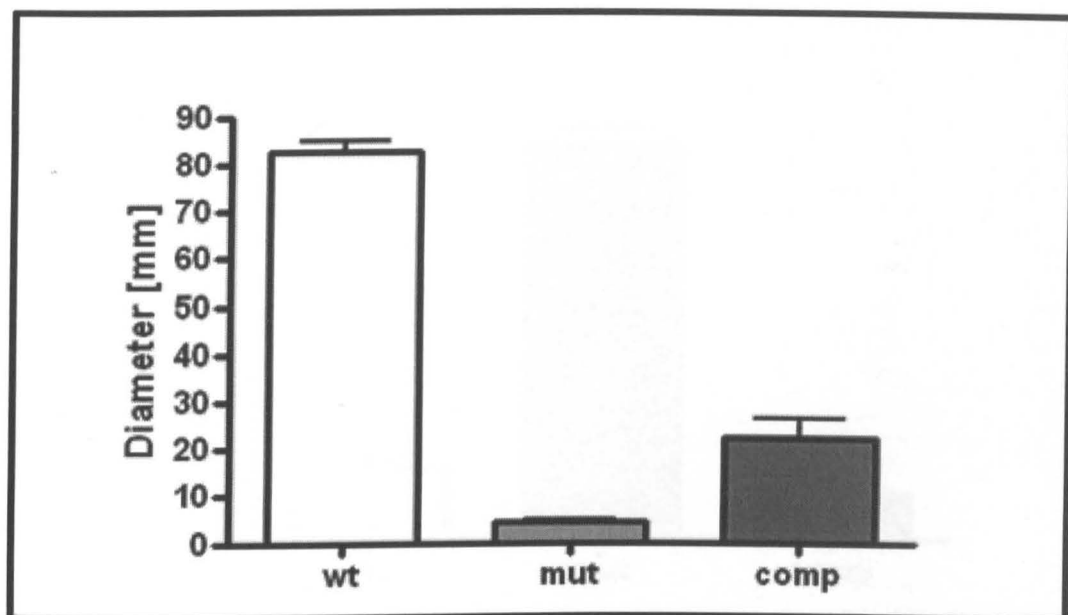


Figure 6.11: The motility of the *C. jejuni* 11168H wild-type strain (wt), the 11168H *rpoN* mutant (mut), as well as the complemented 11168H *rpoN* mutant (comp) determined. After 72 h on soft agar plates, the complemented 11168H *rpoN* mutant showed partial rescue of motility.

6.5.4 The autoagglutination phenotype of the 11168H *rpoN* mutant is complemented by expression of a functional copy of *rpoN*

Previously published data demonstrated that the presence of the flagellum is one of the key factors leading to bacterial aggregation (Golden *et al.*, 2002). As the 11168H *rpoN* mutant is aflagellate, this assay would allow for another rapid assessment of the efficiency of the complementation. The *C. jejuni* 11168H wild-type strain, 11168H *rpoN* mutant as well as complemented 11168H *rpoN* mutant were assessed for their autoagglutination properties as described in section 2.11.2. As expected, the *C. jejuni* 11168H wild-type strain displayed a strong autoagglutinating phenotype, while hardly any autoagglutination was observed for the 11168H *rpoN* mutant (Figure 6.12). Autoagglutination comparable to the wild-type strain was observed for the complemented 11168H *rpoN* mutant, suggesting that re-introduction of a functional copy of the *rpoN* gene into the 11168H *rpoN* mutant restored the wild-type autoagglutination phenotype.

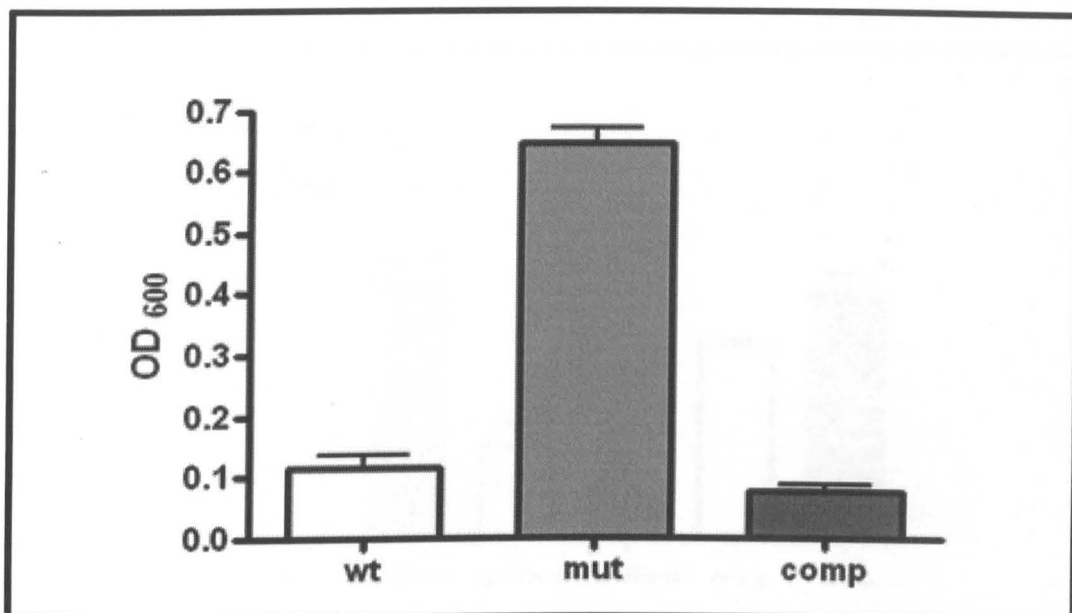
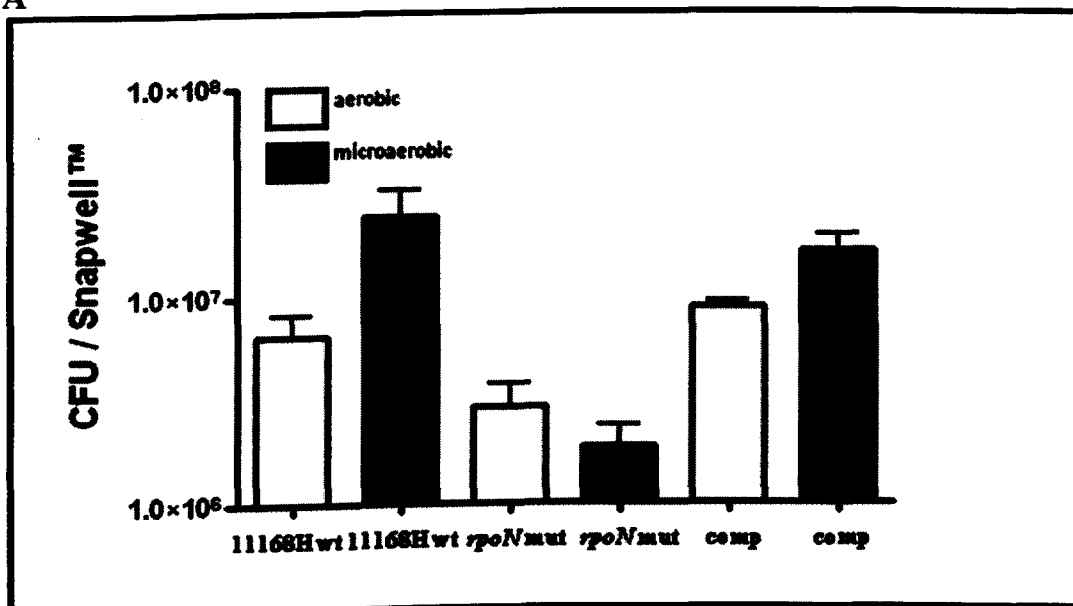


Figure 6.12: The autoagglutination of the *C. jejuni* 11168H wild-type strain (wt), the 11168H *rpoN* mutant (mut), as well as the complemented 11168H *rpoN* mutant (comp) was determined. Complementation of the 11168H *rpoN* mutant fully restored the autoagglutination phenotype.

The results from the motility and autoagglutination experiments suggested that re-insertion of a functional copy of the *rpoN* gene into the chromosome of a 11168H *rpoN* mutant partially restored flagellar filament functionality, as the presence of a functional flagellum has been demonstrated to be essential for autoagglutination of *C. jejuni* (Golden *et al.*, 2002). As the motility was only partially restored, however, it is probable that the function of the flagellar apparatus is only partially complemented.

Nonetheless, the complemented 11168H *rpoN* mutant was co-cultured with Caco-2 IECs in a VDC with either aerobic or microaerobic conditions in the apical compartment for 6 and 24 h and the rate of interaction and invasion determined and compared to those obtained for the *C. jejuni* 11168H wild-type strain and the 11168H *rpoN* mutant.

A



B

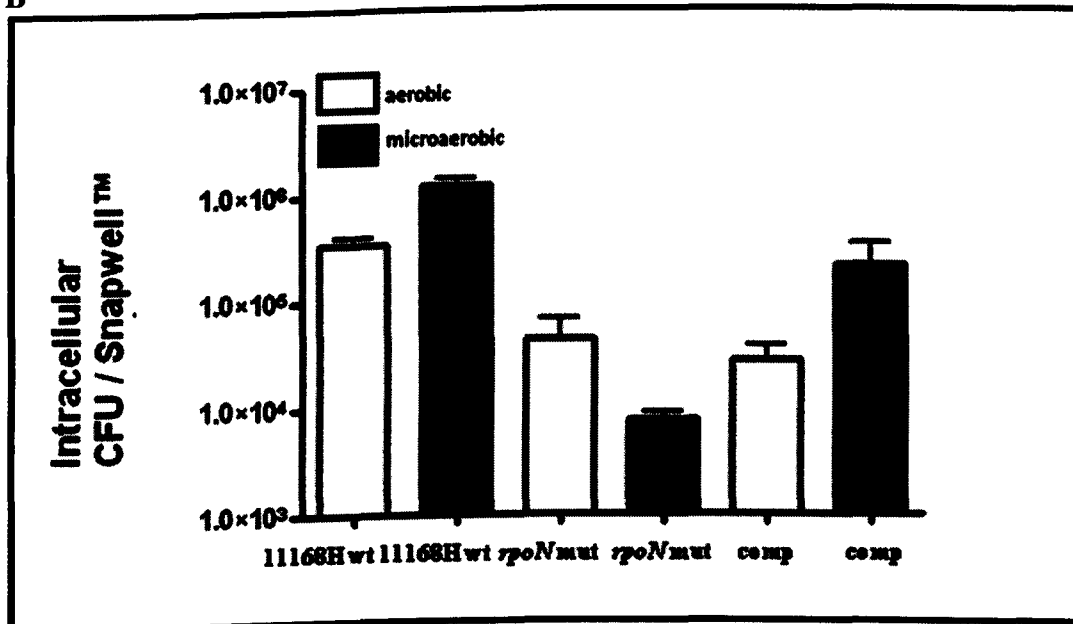
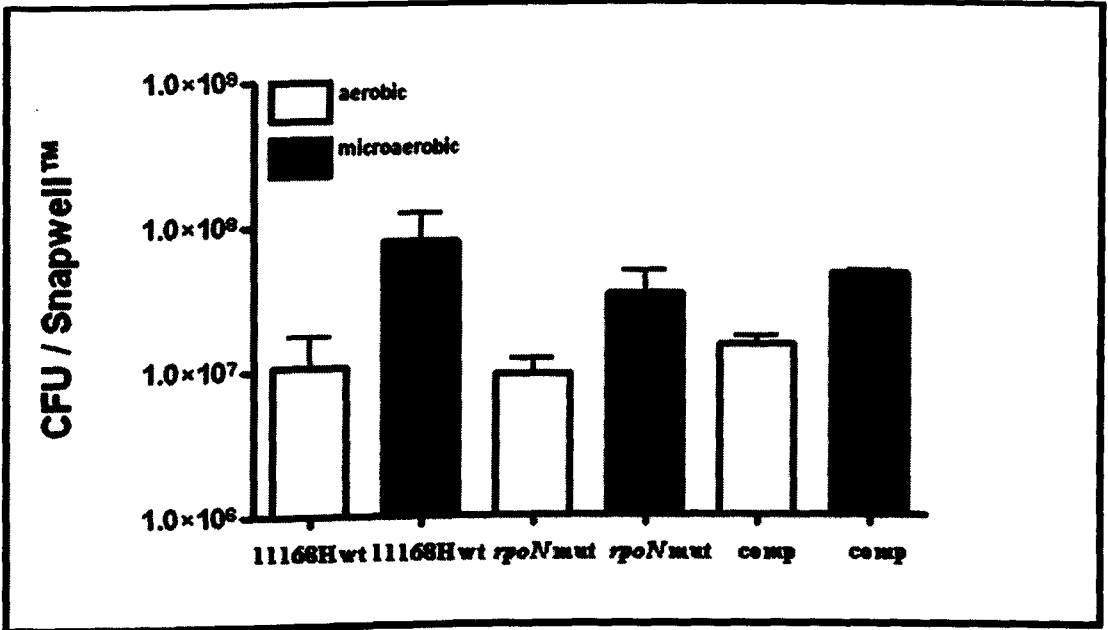


Figure 6.13: *C. jejuni* 11168H wild-type strain (11168H wt), 11168H *rpoN* mutant (*rpoN* mut) and complemented 11168H *rpoN* mutant (comp) bacteria were co-cultured with Caco-2 intestinal epithelial cells (IECs) in a vertical diffusion chamber (VDC) at an MOI of 100:1 for 6 h with either aerobic (white bars) or microaerobic (black bars) conditions in the apical compartment. The numbers of interacting bacteria were determined by lysis of the IECs after co-culturing, serial dilution and plating of the lysates (A). The numbers of intracellular bacteria were determined at both time points by killing of any extracellular bacteria with gentamicin followed by lysis of the IECs and serial dilution and plating of the lysates (B).

A



B

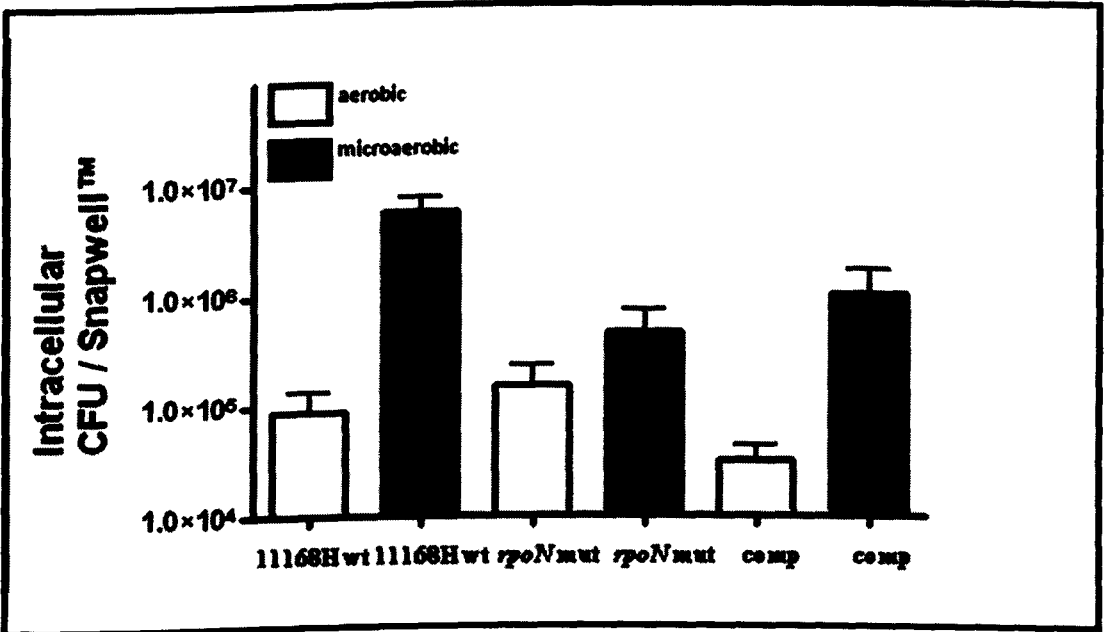


Figure 6.14: *C. jejuni* 11168H wild-type strain (11168H wt), *rpoN* mutant (*rpoN* mut) and complemented 11168H *rpoN* mutant (comp) bacteria were co-cultured with Caco-2 intestinal epithelial cells (IECs) in a vertical diffusion chamber (VDC) at an MOI of 100:1 for 24 h with either aerobic (white bars) or microaerobic (black bars) conditions in the apical compartment. The numbers of interacting bacteria were determined by lysis of the IECs after co-culturing, serial dilution and plating of the lysates (A). The numbers of intracellular bacteria were determined at both time points by killing of any extracellular bacteria with gentamicin followed by lysis of the IECs and serial dilution and plating of the lysates (B).

After 6 h of co-culturing, complementation of the 11168H *rpoN* mutant partially restored wild-type levels of interaction (Figure 6.13A) and invasion (Figure 6.13B). Most importantly, higher numbers of interacting and intracellular complemented 11168H *rpoN* mutant bacteria were found after incubation in the VDC with microaerobic conditions compared to aerobic conditions in the apical compartment (black compared to white bars). This had been observed for the *C. jejuni* 11168H wild-type strain but not for the 11168H *rpoN* mutant. This confirmed the important role for RpoN in increasing the ability of *C. jejuni* to interact and invade IECs under microaerobic compared to aerobic conditions. After 24 h of co-culturing, complementation of the 11168H *rpoN* mutant partially restored wild-type levels of interaction (Figure 6.14A) and invasion (Figure 6.14B).

The reason for the partial complementation of the 11168H *rpoN* mutant interaction and invasion phenotype by reintroducing a functional copy back into the mutant may be due to the fact that, as suggested previously, only partial restoration of motility was achieved. Evidence presented here as well as other studies have highlighted the importance of motility for *C. jejuni* interaction and invasion of IECs, so partial restoration of motility would most likely only lead to partial restoration of the interaction and invasion phenotype. In wild-type *C. jejuni*, *rpoN* expression has been demonstrated to be controlled and enhanced by FlgR (Wosten *et al.*, 2004). In the complemented *rpoN* mutant, however the complemented *rpoN* gene is under a constitutively active promoter, which may not accurately reflect the transcriptional activity of the native *rpoN* gene, in turn leading to expression profiles of genes controlled by RpoN to differ between the wild-type and the complemented 11168H *rpoN* mutant.

6.6 Summary and discussion

The data presented in Chapter 5 demonstrated that co-culturing of *C. jejuni* 11168H wild-type strain with IECs in the VDC with microaerobic conditions in the apical compartment led to a time-dependent increase in the number of bacteria interacting with and invading the IECs. This part of the study investigated the role of bacterial factors involved in mediating this increased interaction and invasion.

Interestingly, a 11168H *ciaB* mutant was shown to interact and invade IECs in the VDC to the same level as the wild-type strain after 24 h of co-culturing, independent of the oxygen conditions in the apical compartment. This is in contrast to previous data that suggests that a *C. jejuni* F38011 *ciaB* mutant is less able to invade IECs than the wild-type (Konkel *et al.*, 1999b) and recent data obtained with this 11168H *ciaB* mutant (Nevada Naz, LSHTM). However, that data has recently been challenged by another study demonstrating no difference between the interaction and invasion of a *C. jejuni* 81-176 *ciaB* mutant and the parental wild-type strain (Novik *et al.*, 2010). The results obtained with a *C. jejuni* 11168H *ciaB* mutant in the VDC support the evidence obtained with a *C. jejuni* 81-176 *ciaB* mutant, suggesting that the originally reported reduced invasive phenotype of the *C. jejuni* F38011 *ciaB* mutant may be a result of strain variation, or possibly even due to polar/non-specific effect, as no complementation of the phenotype of *C. jejuni* F38011 was reported. Further studies of *C. jejuni* *ciaB* mutants, possibly from different strain backgrounds, may shed light on the CiaB conundrum. Additionally, as the original data demonstrating the requirement of CiaB for IEC invasion used relatively short co-culturing times (Konkel *et al.*, 1999a), this may suggest that CiaB is particularly important for the early stage of *C. jejuni* IEC invasion. Therefore, analysis of the 11168H *ciaB* mutant after shorter co-culturing times in the VDC may shed more light on the importance of CiaB. However, due to time and throughput constraints, the 11168H *ciaB* mutant was not further analysed.

Motility has long been implicated to be important for the ability of *C. jejuni* to interact and in particular to invade IECs in classical *in vitro* co-culturing assays (Szymanski *et al.*, 1995). To investigate whether motility was important for interaction and invasion of IECs by *C. jejuni* in the VDC model, two non-motile *C.*

jejuni mutants were analysed. The 11168H *flaA* mutant is unable to produce the main flagellar filament protein FlaA, whilst the 11168H *rpoN* mutant lacks the alternative sigma factor σ^{54} . When co-cultured with Caco-2 IECs in the VDC for 24 h, both mutants showed weak attenuation in regards to interaction with the IECs. However, both the *C. jejuni* 11168H *flaA* and the *rpoN* mutants exhibited lower numbers of intracellular bacteria compared to the *C. jejuni* 11168H wild-type strain after 24 h of co-culturing, particularly when co-cultured with microaerobic conditions in the apical compartment of the VDC. This suggests that the absence of functional flagella inhibits the ability of *C. jejuni* to increase the interaction and invasion of Caco-2 IECs when co-cultured in the VDC with microaerobic conditions in the apical compartment. Intriguingly, the phenotypes of both the 11168H *flaA* and *rpoN* mutants were more pronounced after 6 h of co-culturing in the VDC compared to 24 h of co-culturing. Both mutants showed significantly reduced numbers of interacting and intracellular bacteria compared to the *C. jejuni* 11168H wild-type strain. Interestingly, at this time point, more *flaA* and *rpoN* mutant bacteria were found to be interacting with the IECs after co-culturing in the VDC with aerobic compared to microaerobic conditions in the apical compartment. This supports the hypothesis that increased motility in microaerobic conditions is an important cause of the increased bacterial interaction and invasion. Intriguingly, the phenotype was more apparent after shorter co-culturing times and both mutants appeared to be able to “catch-up” with the wild-type strain after longer co-incubation with the IECs. This suggests that motility is particularly important at the early stages of infection. This observation may also be in part due to the wild-type strain losing some of the motility after longer co-culturing. Alternatively, as both mutants are non-motile and exhibit impaired autoagglutination, it is possible that after longer co-culturing, some of the wild-type bacteria have agglutinated and fallen away from the IECs in the VDC, while the two mutants are still in suspension. This is more pronounced in the VDC model setup, where the IECs are mounted vertically, and the bacteria need to actively swim towards the IECs while being pulled towards the bottom of the VDC by gravity. This is in contrast to the classical tissue culture method, where the IECs are located at the bottom of a tissue culture flask, and even non-motile bacteria down are brought into proximity of the IECs by gravity. In order to investigate this hypothesis, a flagellated and autoagglutinating but non-motile mutant such as a *C. jejuni* 11168H *Cj0248* mutant (Ozan Gundogdu, personal communication) should be

co-cultured with IECs in the VDC and analysed for interaction with and invasion of the IECs.

Compared to the 11168H *flaA* mutant, the 11168H *rpoN* mutant exhibited a more pronounced phenotype, with fewer bacteria interacting with and invading the IECs. This may be due to the fact that while the *flaA* mutant lacks the major flagellar filament protein, the bacteria still possess a truncated flagellum as well as a flagellar hook and a basal body, and is still able to secrete proteins (Konkel *et al.*, 2004). The *rpoN* mutant on the other hand is completely aflagellate, does not possess a flagellar hook and is not able to secrete proteins (Fernando *et al.*, 2007). Furthermore, microarray analysis of gene transcription of a *C. jejuni* 11168H *rpoN* mutant revealed multiple changes, in genes involved in flagellar biosynthesis and structure as well as in non-flagellar genes (Kamal *et al.*, 2007). Therefore, the *rpoN* mutation may have a more global effect on the bacteria, and hence a more severe impact on the ability of the bacteria to interact with the IECs.

Re-introduction of a functional copy of the *rpoN* gene into the chromosome of the 11168H *rpoN* mutant fully restored the autoagglutination phenotype, and partially restored the motility, interaction and invasion phenotypes of the mutant. As RpoN acts as an alternative sigma factor, which has been shown to be controlled by FlgR, it is likely that the constitutive expression of RpoN from the complementation construct is not at the exact same level as it would be in the *C. jejuni* 11168H wild-type strain, resulting in different downstream expression of genes controlled by RpoN, which in turn would lead to a different phenotype. Gene expression analysis of the complemented 11168H *rpoN* mutant would allow further clarification of this point.

Taken together, the data presented here demonstrates the importance of bacterial motility in the interaction with and invasion of IECs by *C. jejuni*. This data also indicates that changed/increased motility under microaerobic conditions is an important factor in the increased interaction and invasion of the IECs reported in the previous Chapter 6. This may well be reflective of the environment of the human intestine, where the conditions are microaerobic or even anaerobic. The findings reported here suggest that *C. jejuni* potentially exhibits stronger motility when inside the human intestine. This would aid the bacteria in swimming through the intestinal contents towards the IECs *in vivo* and therefore facilitate attachment and invasion. As all classical *C. jejuni* IEC co-culturing models used to date have been performed

under aerobic conditions, it is likely that the conditions have been suboptimal for the bacteria, and have hence hampered the discovery of the molecular basis of *C. jejuni* pathogenesis.

Further evidence obtained using the 11168H *rpoN* mutant, which is also non-motile, suggested that bacterial motility was not the sole reason for the increased interaction and invasion of the IECs observed under microaerobic conditions. It remains to be determined which genes are differentially expressed in the 11168H *rpoN* mutant compared to the 11168H *flaA* mutant and thus may have an influence in *C. jejuni* interactions with and invasion of IECs.

Chapter 7

Summary and future studies

7.1 Introduction

Despite the prevalence of *C. jejuni* as a causative agent of human foodborne gastroenteritis, the molecular basis of the organism's pathogenicity is still poorly understood compared with other enteric pathogens. This can mainly be attributed to i: the lack of a convenient small animal model of the disease and ii: certain drawbacks with the commonly used *in vitro* models when applied to the study of *C. jejuni* pathogenesis.

The research reported in this study describes the development of two models to study the pathogenesis of *C. jejuni*. Both models were chosen to more closely mimic the situation encountered by the pathogen *in vivo* in the human intestine. The EVOC model utilised *ex vivo* cultured human intestinal tissue to mimic the complex host tissue encountered by *C. jejuni in vivo*, whilst the VDC model mimics the microaerobic/anaerobic nature of the intestinal lumen.

7.2 EVOC model summary

The EVOC model employed whole primary human intestinal biopsies in order to present the bacteria with a more *in vivo*-like complex tissue, compared to the commonly employed "single cell-type" *in vitro* co-culturing using epithelial or other cell types. This model has previously been used to microscopically study the interaction of *C. jejuni* with human intestinal tissue (Grant *et al.*, 2006, Haddock *et al.*, 2010) as well as to analyse the host innate immune response to *C. jejuni*, demonstrating an increased secretion of the pro-inflammatory cytokine IL-8 in response to *C. jejuni* infection *ex vivo* (MacCallum *et al.*, 2006). In contrast to published data, no increased secretion of IL-8 was detected from tissue co-cultured with *C. jejuni* 11168H and 81-176 wild-type strains. However, co-culturing of the tissue with *C. jejuni* 11168H and 81-176 wild-type strains was demonstrated to lead to increased secretion of several other innate immune response markers, including human beta-defensins hBD-2 and hB-3. This was the first time that these peptides have been demonstrated to be secreted from *ex vivo* infected tissue, and supports previous data demonstrating a role for these peptides in the intestinal epithelial defence against *C. jejuni*. As primary tissue is difficult to obtain, and initial

experiments demonstrated no difference between the two different *C. jejuni* wild-type strains with regards to the response elicited from *ex vivo* infected tissue, it was decided to perform further EVOC experiments with only the *C. jejuni* 11168H wild-type strain. Evidence was obtained that *C. jejuni* 11168H wild-type strain induced a mixed Th1 / Th17 response in the tissue *ex vivo*, supporting data obtained by collaborators at the ICH (Edwards *et al.*, 2010). Furthermore, a 11168H *flaA* mutant lacking the major flagellar filament protein FlaA and a 11168H *neuB1* mutant lacking the sialic acid residue of the LOS were analysed for the induction of hBD-2 and hBD-3. Both mutants elicited similar levels of secretion of hBD-2 and hBD-3 from *ex vivo* cultured human intestinal tissue compared to the *C. jejuni* 11168H wild-type strain, suggesting that the presence of the flagellar filament and the sialic acid residue are not required for the induction of an hBD-2/hBD-3 response from *ex vivo* co-cultured human intestinal tissue.

7.3 EVOC model future work

As discussed at the end of Chapter 3, the EVOC model possesses several limitations such as possible strong person-to-person variation, as well as the problem that any response detected from the whole tissue cannot easily be attributed to a specific cell type within the whole tissue. Fractionation of the tissue followed by individual co-culturing of the fractions with *C. jejuni* could be performed to assess the contribution of specific cell subsets to the *ex vivo* response to *C. jejuni* infection. Furthermore, the mounting of the tissue on foam supports followed by inoculation of the bacteria straight onto the tissue may result in some bacteria being able to gain access to parts of the tissue they would not necessarily be able to *in vivo*, such as the underlying immune cells. Modifying the EVOC model as described in Schuller *et al.*, 2009, where the tissue was mounted between two small plastic discs with only the apical, epithelial side protruding, may be a way to address this issue. Even though the EVOC model cannot be used for high throughput screening, the model should be further employed to study *C. jejuni*-host interactions, as this model offers a closer reflection of the situation encountered by the pathogen *in vivo* regarding the complexity of the host tissue encountered. In terms of *C. jejuni* mutants, bacteria deficient in or possessing modified surface structures such as a more truncated LOS, completely aflagellate mutants such as a σ^{54} (*rpoN*) mutant or mutants possessing flagella yet lacking the *O*-linked glycosylation of the flagellar subunits should be

studied using the EVOC model to further investigate the role of *C. jejuni* surface structures in host-pathogen interactions. Another interesting aspect of the EVOC model that has not been considered in this study is the microscopical analysis of the interaction of different *C. jejuni* strains and mutants with the tissue, analysing the interaction and potentially, tissue- and cell-invasion of the different strains. Additionally, as opposed to analysing secreted components of the host immune response to the *C. jejuni* challenge, potential changes of intracellular signalling molecules such as signal kinase-cascades should be studied.

7.4 VDC model summary

One of the major drawbacks when applying standard *in vitro* models to study *C. jejuni* is the presence of atmospheric levels of oxygen. The VDC model described in Chapters 4-6 uses a Vertical Diffusion Chamber to co-culture *C. jejuni* with human intestinal epithelial cells (IECs). The setup of the model allows for the bacteria to be maintained under microaerobic conditions at the apical surface of the IECs, while supplying oxygen to the IECs from the basolateral side. This mimics the microaerobic/anaerobic environment encountered by *C. jejuni in vivo* in the human intestine more closely than the classical aerobic co-culturing method. A time dependent increase in both the numbers of *C. jejuni* interacting with as well as invading the IECs was detected when the bacteria were co-cultured in the VDC under microaerobic compared to aerobic conditions. Furthermore, an increased, polarised IL-8 response was detected from IECs that had been co-cultured with *C. jejuni* under microaerobic conditions, suggesting that the IECs responded to the increased bacterial interaction and invasion. Microarray studies of the gene expression of the *C. jejuni* 11168H wild-type strain co-cultured with IECs in the VDC with either aerobic or microaerobic conditions in the apical compartment demonstrated only minor differences between the gene expression profiles of *C. jejuni* 11168H co-cultured with IECs in the VDC with either aerobic or microaerobic conditions in the apical compartment. No “classical” *C. jejuni* virulence factors were detected to be differentially expressed under the two conditions, suggesting that i: other *C. jejuni* proteins may be involved in modulating the increased interaction with and invasion of IECs under microaerobic conditions, ii: microarray analysis was not sensitive enough to detect differences in gene expression or iii: the two time points

investigated (6 h and 24 h) were too late to detect important changes in gene expression under microaerobic conditions in the VDC.

Analysis of three defined *C. jejuni* 11168H mutants suggested that bacterial motility was an important factor in the increased interaction and invasion observed under microaerobic conditions, as two non-motile mutants demonstrated less of a “increased interactive and invasive” phenotype when co-cultured under microaerobic conditions, particularly after short co-culturing times. Complementation of one of these mutations restored wild-type levels of interaction and invasion.

7.5 VDC model future work

From the model perspective, the VDC model has proved to possess some clear advantages over aerobic *in vitro* *C. jejuni*-IEC co-culturing models. The environment created by the VDC more closely mimics the environment encountered by *C. jejuni* in the human intestine, and led to a highly significant increase in numbers of bacteria interacting with and invading the IECs compared to control co-culturing experiments performed with aerobic conditions in the apical compartment. This will be useful for further studies of *C. jejuni* host-pathogen interactions. One factor that needs to be considered when using the VDC model is that the long co-culturing times employed for some of the experiments appeared to mask the phenotypes of the 11168H *flaA* and the 11168H *rpoN* mutants. Whether this is due to the altered autoagglutination phenotype of the mutants as discussed in Chapter 6, or *per se* a limitation of the model remains to be elucidated. However, after shorter co-culturing times, the model was able to confirm the reduced invasive phenotype of the two non-motile *C. jejuni* mutants studied. Possibly longer co-culturing experiments should be used for experiments requiring maximal numbers of interacting and intracellular bacteria, while experiments aimed at elucidating differences between *C. jejuni* strains or mutants should be restricted to shorter co-culturing times. Further studies will be required to confirm this suggestion.

Several features regarding the technical/setup side of the VDC model must be considered prior to applying the model to study any pathogenic organism. Firstly, the IECs must be grown to form an impermeable monolayer on Snapwell™ filters. This takes between 14 and 21 days depending on the cell line used and makes the experiments fairly lengthy compared to classical co-culturing experiments performed in tissue culture plates and using IECs grown for between 1 day and 7 day. On the

other hand, only after such a long period of polarisation have the IECs been demonstrated to form a fully polarised monolayer. Such polarised monolayers mimic the situation *in vivo* in the human intestinal epithelium much more closely than the non-polarised, non-confluent IEC lines used in some studies, and as such provide another advantage of the VDC model. Secondly, the Snapwell™ filters are significantly more expensive, with one filter (i.e. one experimental replicate) costing about £4 compared to approximately £1 for a 24-well tissue culture plate (up to 24 experimental replicates or approximately £0.04 per replicate). The main limitation of the VDC, however, is the relatively low throughput. This is mainly due to the availability of VDC chambers and the setup requiring the gas manifold. As such, only relatively low numbers of parallel replicates could be performed in this study, in contrast to the classical tissue culture method, where one or more 24-well or even 96-well plates of IECs can be simultaneously co-cultured with a pathogen. The model is therefore less suitable for large-scale screening experiments or experiments requiring a high number of replicates. Therefore, while the model is better than the classical, aerobic tissue-culture method due to the more closely mimicked *in vivo* situation, especially for bacteria such as *C. jejuni* that have a stringent atmospheric requirement, the model still has certain limitations that need to be taken into account when designing experiments.

The model has proven feasible for studying *C. jejuni* interactions with IECs, but still has certain limitations such as low throughput, and being relatively expensive and laborious compared to classical co-culturing methods. Further investigation should include a more detailed analysis of gene transcription of *C. jejuni* co-cultured under both atmospheric conditions by high-throughput mRNA sequencing (RNASeq). This will give a better understanding of the effects of co-culturing than the data obtained using microarrays. However, the limitation will still be the low quantities of RNA available from the co-culturing experiments; pooling of several samples from different VDCs might address this. Whilst the relatively low throughput of the VDC model limits the potential for high throughput screening experiments, it would still be interesting to assess further *C. jejuni* wild-type strains and mutants using the VDC model, especially if data obtained by RNASeq highlighted any novel, uncharacterised genes as being involved in the increased interaction and invasion. As more intracellular *C. jejuni* were recovered after microaerobic co-culturing with the IECs, the numbers of intracellular bacteria may be sufficient to isolate enough

mRNA for microarray or RNASeq analysis of the intracellular bacteria. Furthermore, the intracellular fate of *C. jejuni* could be studied by microscopy using markers for different endosomal pathways, a method that was previously hampered by the low numbers of intracellular *C. jejuni* in the classical co-culturing model.

The effect of the increase in interacting and intracellular *C. jejuni* on the IECs should be investigated in more detail. Analysis of the effect of the bacteria on intracellular kinase cascades should be assessed. Additionally, a multiplex ELISA should be used to analyse the host innate immune response with regards to further cytokines or defensin peptides. Alternatively, as the levels of cytokines or defensin peptides secreted by the IECs may be below the detection limit of an ELISA method, the transcriptional activity of genes related to the innate immune response should be analysed either by microarray or even by RNASeq.

Combining the two models by setting up human intestinal tissue in the VDC with microaerobic conditions in the apical compartment was contemplated, however, problems with the tissue integrity of the whole biopsy under these experimental conditions were anticipated (Keith Lindley and Stephanie Schüller, personal communications), and in light of i: the difficulty of obtaining human intestinal tissue and ii: the relative low throughput of both the models, this setup was not pursued. However, further investigation into this setup would certainly be merited.

Taken together, the work presented in this thesis describes the development of two models for studying the host-pathogen interactions of *C. jejuni*. Both models aimed to create an environment more closely mimicking the environment encountered by *C. jejuni in vivo* in the human intestine. The EVOC model demonstrated for the first time that infection of human intestinal tissue *ex vivo* with *C. jejuni* led to the secretion of hBD-2 and hBD-3. Additionally, the model demonstrated for the first time that infection of human intestinal tissue *ex vivo* with *C. jejuni* led to the development of a Th-1/Th-17 response. The VDC model demonstrated that co-culturing of IECs with *C. jejuni* in the VDC with microaerobic conditions in the apical compartment lead to a highly significant increase of both interacting and intracellular bacteria. The VDC model also demonstrated an increased, polarised innate immune response by the IECs. Further studies using these two models will

shed new light onto the hitherto relatively elusive molecular pathogenesis mechanisms of the world's most prevalent foodborne pathogen, *C. jejuni*.

8 Appendices

8.1 Appendix I: *C. jejuni* 11168H genes identified by microarray analysis to be up-regulated after 6 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment

ORF number	Normalised data	Re-annotation
<i>Cj1511c</i>	2.3	<i>fdhA</i> putative formate dehydrogenase large subunit
<i>Cj1705c</i>	2.0	binds third domain of 23S rRNA and protein L29; part of exit tunnel
<i>Cj1186c</i>	2.0	<i>petA</i> putative ubiquinol-cytochrome C reductase iron-sulfur subunit
<i>Cj1325</i>	1.9	putative methyltransferase
<i>Cj0347</i>	1.9	<i>rpF</i> N-(5'-phosphoribosyl) anthranilate isomerase
<i>Cj0414</i>	1.8	putative oxidoreductase subunit
<i>Cj0473</i>	1.7	<i>nusG</i> transcription antitermination protein NusG
<i>Cj0207</i>	1.6	translation initiation factor IF-3
<i>Cj1591</i>	1.6	50S ribosomal protein L36
<i>Cj0330c</i>	1.6	50S ribosomal protein L32
<i>Cj1439c</i>	1.6	<i>glf</i> UDP-galactopyranose mutase part of the capsule locus
<i>Cj0859c</i>	1.6	hypothetical protein
<i>Cj1267c</i>	1.6	<i>hydA</i> Ni/Fe-hydrogenase small chain
<i>Cj0581</i>	1.5	dinucleoside polyphosphate hydrolase
<i>Cj0329c</i>	1.5	putative glycerol-3-phosphate acyltransferase PlsX
<i>Cj1027c</i>	1.5	<i>gyrA</i> DNA gyrase subunit A
<i>Cj0007</i>	1.5	<i>gltB</i> glutamate synthase large subunit
<i>Cj1028c</i>	1.5	putative purine/pyrimidine phosphoribosyltransferase
<i>Cj0397c</i>	1.5	hypothetical protein
<i>Cj0519</i>	1.5	putative rhodanese-like domain protein
<i>Cj0995c</i>	1.5	<i>hemB</i> delta-aminolevulinic acid dehydratase
<i>Cj0802</i>	1.5	cysteinyl-tRNA synthetase
<i>Cj0315</i>	1.5	putative HAD-superfamily hydrolase
<i>Cj0398</i>	1.4	<i>gatC</i> aspartyl/glutamyl-tRNA amidotransferase subunit C
<i>Cj0462</i>	1.4	hypothetical protein
<i>Cj1223c</i>	1.4	<i>dccR</i> two-component response regulator
<i>Cj1172c</i>	1.4	hypothetical protein
<i>Cj0953c</i>	1.4	<i>urH</i> de novo purine biosynthesis

<i>Cj0961c</i>	1.4	50S ribosomal protein L34
<i>Cj0092</i>	1.4	putative periplasmic protein
<i>Cj0789</i>	1.4	<i>cca</i> putative multifunctional Cca protein RNA synthesis
<i>Cj1621</i>	1.3	putative periplasmic protein
<i>Cj0271</i>	1.3	bacterioferritin comigratory protein homolog
<i>Cj0348</i>	1.3	<i>trpB</i> tryptophan synthase subunit beta
<i>Cj0320</i>	1.3	<i>fliH</i> flagellar assembly protein H
<i>Cj0146c</i>	1.3	<i>trxB</i> thioredoxin reductase
<i>Cj0498</i>	1.3	<i>trpC</i> indole-3-glycerol-phosphate synthase
<i>Cj0237</i>	1.3	<i>cynT</i> carbonic anyhydrase
<i>Cj0976</i>	1.3	putative methyltransferase
<i>Cj0891c</i>	1.2	<i>serA</i> D-3-phosphoglycerate dehydrogenase
<i>Cj0934c</i>	1.2	putative sodium:amino-acid symporter family protein
<i>Cj0055c</i>	1.2	hypothetical protein
<i>Cj0552</i>	1.2	hydrophobic protein

8.2 Appendix II: *C. jejuni* 11168H genes identified by microarray analysis to be down-regulated after 6 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment

ORF number	Normalised data	Re-annotation
<i>Cj0304c</i>	1.3	<i>bioC</i> putative biotin synthesis protein
<i>Cj0522</i>	1.3	putative Na ⁺ /Pi cotransporter protein
<i>Cj1634c</i>	1.3	chorismate synthase
<i>Cj0410</i>	1.4	fumarate reductase iron-sulfur protein
<i>Cj0486</i>	1.4	possible L-fucose symporter
<i>Cj0303c</i>	1.4	<i>modA</i> putative molybdate-binding lipoprotein
<i>Cj1666c</i>	1.4	putative periplasmic protein
<i>Cj0988c</i>	1.5	very hypothetical protein
<i>Cj1079</i>	1.5	putative periplasmic protein
<i>Cj1134</i>	1.5	<i>htrB</i> lipid A biosynthesis lauroyl acyltransferase
<i>Cj0728</i>	1.6	putative periplasmic protein
<i>Cj0590</i>	1.6	putative SAM-dependent methyltransferase
<i>Cj0737</i>	1.6	putative hemagglutination activity domain-containing protein
<i>Cj0560</i>	1.7	putative MATE family transport protein
<i>Cj1521c</i>	1.7	putative CRISPR-associated protein
<i>Cj0290c</i>	1.8	pseudogene (partial <i>glpT</i>)
<i>Cj1581c</i>	1.9	putative peptide ABC transporter ATP-binding protein
<i>Cj0302c</i>	2.0	putative molybdenum-pterin binding protein
<i>Cj0141c</i>	2.0	putative ABC transporter integral membrane protein
<i>Cj0422c</i>	2.0	putative H-T-H containing protein
<i>Cj1378</i>	2.2	<i>selA</i> selenocysteine synthase
<i>Cj1352</i>	2.3	<i>ceuB</i> enterochelin uptake permease
<i>Cj1629</i>	2.5	<i>exbD2</i> putative <i>exbD</i> /tolR family transport protein
<i>Cj1393</i>	2.6	<i>metC'</i> putative cystathionine beta-lyase

8.3 Appendix III: *C. jejuni* 11168H genes identified by microarray analysis to be upregulated after 24 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment

ORF number	Normalised data	Re-annotation
<i>Cj1325</i>	2.8	putative methyltransferase
<i>Cj1176c</i>	2.2	<i>tatA</i> Sec-independent protein translocase
<i>Cj0985c</i>	2.1	<i>hipO</i> hippurate hydrolase
<i>Cj0073c</i>	2.1	hypothetical protein
<i>Cj0922c</i>	2.1	<i>pebC</i> amino-acid ABC transporter ATP-binding protein
<i>Cj1326</i>	2.0	hypothetical protein
<i>Cj0347</i>	2.0	<i>trpF</i> N-(5'-phosphoribosyl)anthranilate isomerase
<i>Cj0592c</i>	1.9	putative periplasmic protein
<i>Cj0920c</i>	1.9	putative ABC-type amino-acid transporter permease
<i>Cj0628</i>	1.8	putative lipoprotein
<i>Cj0127c</i>	1.8	<i>accD</i> acetyl-CoA carboxylase subunit beta
<i>Cj1558</i>	1.8	hypothetical protein
<i>Cj1273c</i>	1.8	<i>rpoZ</i> DNA-directed RNA polymerase subunit omega
<i>Cj0346</i>	1.6	<i>trpD</i> anthranilate synthase component II
<i>Cj1172c</i>	1.6	hypothetical protein
<i>Cj1668c</i>	1.6	putative periplasmic protein
<i>Cj1560</i>	1.6	pseudogene
<i>Cj0802</i>	1.6	<i>cysS</i> cysteinyl-tRNA synthetase
<i>Cj0813</i>	1.6	<i>kdsB</i> 3-deoxy-manno-octulosonate cytidylyl-transferase
<i>Cj0459c</i>	1.6	hypothetical protein
<i>Cj0029</i>	1.6	<i>ansA</i> cytoplasmic L-asparaginase
<i>Cj1321</i>	1.6	putative transferase LSO
<i>Cj1193c</i>	1.6	putative periplasmic protein
<i>Cj0152c</i>	1.6	hypothetical protein
<i>Cj1345c</i>	1.5	putative periplasmic protein
<i>Cj0244</i>	1.5	<i>rpmI</i> 50S ribosomal protein L35
<i>Cj1186c</i>	1.5	<i>petA</i> putative ubiquinol-cytochrome C reductase iron-sulfur subunit
<i>Cj1639</i>	1.5	NifU protein

<i>Cj1503c</i>	1.5	<i>putA</i> putative / Δ -1-pyrroline-5-carboxylate dehydrogenase
<i>Cj1705c</i>	1.5	50S ribosomal protein L23
<i>Cj0960c</i>	1.5	putative ribonuclease P protein component
<i>Cj0330c</i>	1.5	50S ribosomal protein L32
<i>Cj0898</i>	1.5	putative histidine triad (HIT) family protein
<i>Cj1603</i>	1.5	<i>hisF</i> imidazole glycerol phosphate synthase subunit HisF
<i>Cj1681c</i>	1.5	<i>cysQ</i> CysQ protein
<i>Cj0685c</i>	1.5	<i>cipA</i> invasion protein CipA
<i>Cj0092</i>	1.4	putative periplasmic protein
<i>Cj1616</i>	1.4	<i>chuC</i> putative hemin uptake system ATP-binding protein
<i>Cj0912c</i>	1.4	<i>cysM</i> cysteine synthase
<i>Cj1207c</i>	1.4	putative lipoprotein thiredoxin
<i>Cj0771c</i>	1.4	putative NLPA family lipoprotein
<i>Cj0129c</i>	1.4	outer membrane protein
<i>Cj0070c</i>	1.4	hypothetical protein
<i>Cj1719c</i>	1.4	<i>euA</i> 2-isopropylmalate synthase
<i>Cj0371</i>	1.4	hypothetical protein
<i>Cj0234c</i>	1.4	<i>frr</i> ribosome recycling factor
<i>Cj0507</i>	1.4	<i>maf</i> Maf-like protein
<i>Cj1541</i>	1.4	LamB/YcsF family protein
<i>Cj0979c</i>	1.4	putative secreted nuclease
<i>Cj0362</i>	1.3	putative integral membrane protein
<i>Cj0914c</i>	1.3	CiaB protein
<i>Cj0089</i>	1.3	putative lipoprotein
<i>Cj1537c</i>	1.3	<i>acs</i> acetyl-CoA synthetase
<i>Cj0407</i>	1.3	<i>lgt</i> prolipoprotein diacylglyceryl transferase
<i>Cj0290c</i>	1.3	pseudogene
<i>Cj0489</i>	1.3	<i>ald'</i> putative aldehyde dehydrogenase N-terminus
<i>Cj0424</i>	1.3	putative acidic periplasmic protein
<i>Cj1508c</i>	1.3	<i>fdhD</i> formate dehydrogenase accessory protein
<i>Cj1543</i>	1.3	putative allophanate hydrolase subunit 2
<i>Cj0854c</i>	1.3	putative periplasmic protein
<i>Cj0466</i>	1.3	<i>nssR</i> transcriptional regulator
<i>Cj0473</i>	1.3	<i>nusG</i> transcription antitermination protein NusG
<i>Cj0682</i>	1.2	hypothetical protein
<i>Cj1115c</i>	1.2	putative phosphatidylserine decarboxylase-related protein
<i>Cj0068</i>	1.2	<i>pspA</i> protease
<i>Cj1254</i>	1.2	hypothetical protein
<i>Cj0099</i>	1.2	<i>birA</i> biotin--protein ligase
<i>Cj0808c</i>	1.2	hypothetical protein

<i>Cj0408</i>	1.2	<i>frdC</i> fumarate reductase cytochrome b-556 subunit
<i>Cj0460</i>	1.2	<i>nusA</i> transcription elongation factor NusA
<i>Cj1504c</i>	1.2	<i>seldD</i> putative selenide,water dikinase
<i>Cj0734c</i>	1.1	<i>hisJ</i> histidine-binding protein precursor
<i>Cj0306c</i>	1.1	<i>bioF</i> 8-amino-7-oxononanoate synthase

8.4 Appendix IV: *C. jejuni* 11168H genes identified by microarray analysis to be down-regulated after 24 h of co-culturing with Caco-2 IECs in the VDC with microaerobic conditions in the apical compartment

ORF number	Normalised data	Re-annotation
<i>Cj1680c</i>	1.1	putative periplasmic protein
<i>Cj1447c</i>	1.1	<i>kpsT</i> capsule polysaccharide export ATP-binding protein
<i>Cj0642</i>	1.2	<i>recN</i> putative DNA repair protein
<i>Cj0843c</i>	1.1	putative secreted transglycosylase
<i>Cj1234</i>	1.1	<i>glyS</i> glycyl-tRNA synthetase subunit bet
<i>Cj0847</i>	1.1	<i>psd</i> phosphatidylserine decarboxylase
<i>Cj1241</i>	1.1	putative MFS transport protein arabinose efflux
<i>Cj1040c</i>	1.2	putative MFS transport protein cyanate transport
<i>Cj1484c</i>	1.2	putative membrane protein
<i>Cj1600</i>	1.2	<i>hisH</i> imidazole glycerol phosphate synthase subunit HisH
<i>Cj0272</i>	1.2	hypothetical protein
<i>Cj0343c</i>	1.2	putative integral membrane protein
<i>Cj0238</i>	1.2	putative mechanosensitive ion channel family protein
<i>Cj0652</i>	1.2	<i>pbpC</i> penicillin-binding protein
<i>Cj0574</i>	1.2	<i>ilvI</i> acetolactate synthase 3 catalytic subunit
<i>Cj0319</i>	1.2	<i>fliG</i> flagellar motor switch protein G
<i>Cj1655c</i>	1.2	<i>nhaA1</i> Na(+)/H(+) antiporter
<i>Cj0720c</i>	1.2	<i>flaC</i> flagellin
<i>Cj0850c</i>	1.3	putative MFS transport protein Sugar transporter
<i>Cj0265c</i>	1.3	putative cytochrome C-type haem-binding periplasmic protein
<i>Cj0849c</i>	1.3	hypothetical protein
<i>Cj0549</i>	1.3	<i>fliS</i> flagellar protein FliS
<i>Cj1294</i>	1.3	<i>pseC</i> C4 aminotransferase
<i>Cj0453</i>	1.3	<i>thiC</i> thiamine biosynthesis protein ThiC
<i>Cj0931c</i>	1.3	<i>argH</i> argininosuccinate lyase
<i>Cj0174c</i>	1.3	<i>cfbpB</i> putative iron-uptake ABC transporter permease
<i>Cj0241c</i>	1.3	putative iron-binding protein
<i>Cj1440c</i>	1.3	putative sugar transferase capsule locus
<i>Cj0846</i>	1.3	putative metallophosphoesterase
<i>Cj0293</i>	1.3	<i>surE</i> stationary phase survival protein SurE
<i>Cj1685c</i>	1.4	<i>bioB</i> biotin synthase
<i>Cj1669c</i>	1.4	DNA ligase

<i>Cj1686c</i>	1.4	<i>topA</i> DNA topoisomerase I
<i>Cj1277c</i>	1.4	putative ABC transporter ATP-binding protein
<i>Cj1555c</i>	1.4	hypothetical protein
<i>Cj0263</i>	1.4	<i>zupT</i> zinc transporter ZupT
<i>Cj0053c</i>	1.4	<i>mnmA</i> tRNA-specific 2-thiouridylase MnmA
<i>Cj1425c</i>	1.5	<i>hddA</i> putative D-glycero-D-manno-heptose 7-phosphate kinase capsule locus
<i>Cj0081</i>	1.5	<i>cydA</i> cytochrome bd oxidase subunit I
<i>Cj1119c</i>	1.5	<i>pglG</i> putative integral membrane protein
<i>Cj0316</i>	1.5	<i>pheA</i> chorismate mutase/prephenate dehydratase
<i>Cj1085c</i>	1.6	<i>mfd</i> transcription-repair coupling factor
<i>Cj1224</i>	1.6	putative iron-binding protein
<i>Cj0303c</i>	1.6	<i>modA</i> putative molybdate-binding lipoprotein
<i>Cj0456c</i>	1.6	hypothetical protein
<i>Cj1138</i>	1.6	putative glycosyltransferase LOS
<i>Cj0439</i>	1.6	<i>dhC</i> putative succinate dehydrogenase subunit C
<i>Cj1098</i>	1.7	<i>pyrB</i> aspartate carbamoyltransferase catalytic subunit
<i>Cj0111</i>	1.7	periplasmic protein
<i>Cj1529c</i>	1.7	<i>purM</i> phosphoribosylaminoimidazole synthetase
<i>Cj0082</i>	1.7	<i>cydB</i> cytochrome bd oxidase subunit II
<i>Cj0480c</i>	1.7	H-T-H motif
<i>Cj0437</i>	1.7	<i>sdhA</i> succinate dehydrogenase flavoprotein subunit
<i>Cj0198c</i>	1.7	recombination factor protein RarA
<i>Cj0501</i>	1.8	pseudo
<i>Cj0032</i>	1.9	putative type IIS restriction /modification enzyme, C-terminal half
<i>Cj1170c</i>	2.1	<i>omp50</i> 50 kda outer membrane protein precursor
<i>Cj0017c</i>	2.4	<i>dsbI</i> disulphide bond formation protein
<i>Cj0799c</i>	2.9	<i>ruvA</i> Holliday junction DNA helicase RuvA

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